Rapamycin-FKBP Inhibits Cell Cycle Regulators of Proliferation in Vascular Smooth Muscle Cells

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Abstract Multiple growth factors can stimulate quiescent vascular smooth muscle cells to exit from G0 and reenter the cell cycle. The macrolide antibiotic rapamycin, bound to its cytosolic receptor FKBP, is an immunosuppressant and a potent inhibitor of cellular proliferation. In the present study, the antiproliferative effects of rapamycin on human and rat vascular smooth muscle cells were examined and compared with the effects of a related immunosuppressant, FK520. In vascular smooth muscle cells, rapamycin, at concentrations as low as 1 ng/mL, inhibited DNA synthesis and cell growth. FK520, an analogue of the immunosuppressant FK506, is structurally related to rapamycin and binds to FKBP but did not inhibit vascular smooth muscle cell growth. Molar excesses of FK520 blocked the antiproliferative effects of rapamycin, indicating that the effects of rapamycin required binding to FKBP. Rapamycin-FKBP inhibited retinoblastoma protein phosphorylation at the G1/S transition. This inhibition of retinoblastoma protein phosphorylation was associated with a decrease in $p33^{cdk2}$ kinase activity. These observations suggest that rapamycin, but not FK520, inhibits vascular smooth muscle cell proliferation by reducing cell-cycle kinase activity. (*Circ Res.* 1995;76:412-417.)

Key Words • immunophilin • accelerated arteriosclerosis • antiproliferation • transplantation • FK506

Abnormal vascular smooth muscle cell (VSMC) proliferation is involved in restenosis following percutaneous transluminal angioplasty (PTCA) and accelerated arteriosclerosis after cardiac transplantation.[1-3] Restenosis occurs after ≈30% to 40% of the procedures,[1,4] limiting the utility of PTCA. Accelerated arteriosclerosis in coronary arteries of the donor heart is a major factor limiting long-term survival of cardiac transplant recipients.[3,5,6] Common to both pathological processes is an injury to the vascular endothelial cell barrier resulting in activation of VSMC proliferation. Multiple signaling pathways can trigger a proliferative response in VSMC. The complexity of cell growth signaling has made it difficult to achieve adequate control of VSMC proliferation in patients.

Much attention has focused on understanding the mechanisms underlying the proliferative response in VSMC. It has been proposed that identifying the regulators of this proliferative response in VSMC may lead to therapeutic strategies aimed at blocking or inhibiting VSMC growth. After deendothelialization of arteries by mechanical injury during PTCA, or by an immune mechanism in transplant recipients, VSMC leave their quiescent state (G0/G1) and enter the cell cycle. Recent studies have shown that early response genes including c-*fos* and c-*myc* are induced after exit from G0.[7,8] Cell-cycle kinases including $p34^{cdc2}$ and mitogen-activated protein kinase homologues appear to be involved in signaling VSMC growth, leading to induction of early response genes.[9] On the other hand, transforming growth factor-β_1 inhibits smooth muscle cells causing a G1 arrest[10,11] that is associated with a decrease in $p34^{cdk2}$ kinase activity.[12] These and other similar observations have led a number of investigators to focus on cell-cycle regulators as potential therapeutic targets for inhibiting VSMC proliferation. For example, antisense oligonucleotides to c-*myc*, c-*myb*, c-*fos*, cyclin A, $p34^{cdc2}$ kinase, and proliferating cell nuclear antigen have been used with varying degrees of success to inhibit VSMC proliferation.[11,13,14]

Recent studies in a rat heart transplantation model have suggested that the macrolide antibiotic FK506, currently used as an immunosuppressant after some types of organ transplant, may accelerate transplant coronary arteriosclerosis.[15-17] In animal models, rapamycin, also a macrolide antibiotic, appears to retard the development of accelerated arteriosclerosis after allograft transplantation and restenosis following mechanical injury.[15,18-20]

In the present study, we sought to compare the effects of rapamycin and FK520 on VSMC proliferation. We found that rapamycin, but not FK520, inhibits cell growth in both human and rat VSMC. This inhibition of cell growth by rapamycin was associated with decreases in cell-cycle kinase activity at the G1/S and G2/M transitions. Phosphorylation of retinoblastoma protein (pRb), a marker for cell-cycle progression, was also reduced. Our data suggest that inhibition of cell-cycle kinases by rapamycin contributes to its potent antiproliferative effects in VSMC. Moreover, because the mechanism of the antiproliferative effect of rapamycin involves inhibition of cell-cycle kinases, rapamycin should block VSMC growth regardless of the stimulus that initiates the VSMC proliferative response. FK520, an analogue of FK506, nonsignificantly accelerated VSMC growth and increased the activity of the cell-cycle regu-

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lators. Thus, compared with FK520, rapamycin has antiproliferative properties that might make it a better choice for use in cardiac transplant recipients in whom VSMC proliferation is a potentially serious problem.

Materials and Methods

Reagents

Rapamycin was a gift from Wyeth-Ayerst Research Laboratory (Dr Suren Sehgal), and FK520 (an FK506 analogue) was provided by Dr John Siekierka (Merck). [^{3}H]Thymidine was from NEN, and polyclonal anti-p34^{cdc2} kinase antibody was a gift from Dr Hiroaki Kiyokawa (Memorial Sloan-Kettering Cancer Institute). Polyclonal antibodies to cyclin D (raised against the C-terminal domain of human cyclin D) and cdk2 (raised against a synthetic peptide in the C-terminal domain of human cdk2) were from Upstate Biotechnology Inc. pRb antibody was from Pharmingen.

Cell Culture

Rat aortic smooth muscle cells (RASM, isolation No. 1120)[21-23] and human aortic smooth muscle cells were gifts from Dr Mark Taubman (Mount Sinai School of Medicine). RASM (passages 8 to 11) were cultured in Dulbecco's modified essential medium (DMEM) plus 20% fetal calf serum (FCS, GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin as previously described.[21] Medium was changed every 48 hours. When cultured in 20% FCS, RASM double approximately every 16 to 20 hours. Human aortic smooth muscle cells from ascending aorta obtained from the donor at the time of cardiac transplantation were cultured in DMEM plus 20% FCS. After plating, rapamycin (100 ng/mL) and FK520 (100 ng/mL) were added directly to DMEM. Cell proliferation analyses were performed by counting triplicate plates at the indicated times during a 7-day period by using a Coulter counter. Cell viability was determined with trypan blue stain for each experiment. Results represent the mean values from three separate experiments; error bars represent the standard error of the mean.

DNA Synthesis

For determination of DNA synthesis, [^{3}H]thymidine incorporation was measured, and microcultures of 5000 cells were established in quadruplicates in flat-bottom 96-well microtiter plates in the presence and absence of varying concentrations of drugs. After 48 hours, each culture was pulsed with 1 μCi [^{3}H]thymidine and harvested 16 to 20 hours later by using a Cambridge Technology PHD Harvester. [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter. The competition experiment with FK520 was performed with 2 ng/mL rapamycin and concentrations of FK520 between 2 and 500 ng/mL.

Flow Cytometric Analysis

Cells were treated with either 100 ng/mL rapamycin or FK520 for 24 hours, harvested, and washed in ice-cold phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored overnight at 4°C before analysis. Cells were then washed once with ice-cold PBS treated with RNAse (1 hour at 37°C, 500 U/mL). Cellular DNA was stained with propidium iodide (50 μg/mL). Cell-cycle determination was performed by using a Coulter analyzer. Results represent a minimum of 3000 cells assayed for each determination.

Preparation of Cellular Lysates

RASM growing in log phase were plated at ≈30% confluence. After 24 hours in DMEM+20% FCS, plates were washed twice with PBS and transferred to DMEM+0.5% FCS for 72 hours to achieve quiescence. Plates were then stimulated with 20% FCS and treated with either no drugs (control), 100 ng/mL rapamycin, or 100 ng/mL FK520. After the indicated time period, plates were washed twice with ice-cold PBS, and cell lysates were prepared using Rb lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L NaF, 0.2 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 0.5% Nonidet P-40). Cells were scraped off the bottom of the plates, and lysates were rocked for 1 hour at 4°C. Lysates were stored at −70°C. Protein concentration was measured by using the Bradford reagent (Bio-Rad), with bovine serum albumin used as a standard.

Determination of Cyclin-Dependent Kinase Activities

Activities of p34^{cdc2} and p33^{cdk2} kinases were analyzed essentially as described previously,[24] with some modifications. Protein extracts (100 μg) were diluted to 500 μL with RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). RASM lysates were immunoprecipitated with either anti-p34^{cdc2} kinase C-terminus–specific antiserum[25] or a human polyclonal anti-p33^{cdk2} kinase antibody. Protein A–Sepharose beads (20 μL) were added and gently rocked for 1 hour at 4°C. Samples were centrifuged and washed twice with RIPA buffer, twice with RIPA without NaCl, and twice with kinase assay buffer (mmol/L: Tris-HCl 50, pH 7.4, $MgCl_2$ 10, and dithiothreitol 1). Phosphorylation was carried out in 25 μL of kinase buffer with the addition of 0.1 mg/mL of histone H1 (Boehringer Mannheim) and 50 μCi [γ-^{32}P]ATP for 15 minutes at 28°C. The reaction was terminated with the addition of 6 μL of 6× Laemmli's sample loading buffer and boiled for 5 minutes. Samples (15 μL) were analyzed on a 12% SDS-polyacrylamide gel. Gels were dried for 2 hours and analyzed by using [γ-^{32}P]ATP standards and a phosphorimager.

Measurement of Retinoblastoma Protein Levels

Protein extracts (50 μg) were size-fractionated on 7.5% gels and transferred to nitrocellulose overnight at 60 V. Filters were blocked in PBS containing 0.1% Tween-20 (PBS-T) and 5% dry milk for 1 hour at 30°C, followed by incubation overnight with anti-pRb antibody (1/1000 dilution) at 4°C. The filters were washed with PBS-T, then incubated with the secondary antibody conjugated to peroxidase (goat anti-mouse IgG) for 1 hour at 4°C, and washed; signals were detected by using the chemiluminescence detection system (ECL) followed by exposure to Kodak XAR film. Autoradiographic signals were quantified by scanning the gels by use of a Macintosh computer with ADOBE PHOTOSHOP and IMAGE 1.44 software. The ratio of hyperphosphorylated to hypophosphorylated pRb was calculated for each time point and plotted. Results are shown for a representative experiment. This experiment was repeated three times, and similar results were obtained each time.

Measurement of Cyclin-Dependent Kinase and Cyclin Protein Levels

Protein extracts (50 μg) were electrophoresed on separate 12% SDS-polyacrylamide gels and transferred to nitrocellulose overnight at 45 V. Filters were blocked in PBS containing 0.1% Tween 20 and 5% dry milk for 1 hour at room temperature, followed by incubation overnight with anti-p34^{cdc2} antibody (1/1000 dilution), anti-p33^{cdk2} antibody (2.5 μg/mL), or anti-cyclin D antibody (2.5 mg/mL). The filters were then washed, incubated with goat anti-rabbit IgG conjugated to peroxidase for 1 hour at 4°C, and washed again; signals were detected by using the chemiluminescence detection system (Bio-Rad) and exposed to Kodak XAR films. Results are shown for representative experiments. These experiments were repeated three times, and similar results were obtained each time.

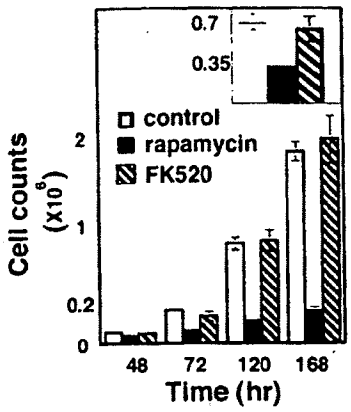

FIG 1. Bar graph showing the time course for rapamycin-induced inhibition of cultured rat aortic smooth muscle cell (RASM) proliferation. The inset shows similar data for human aortic smooth muscle cells at 72 hours. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. The results are expressed in mean cell numbers of triplicate plates; error bars represent standard deviation of the mean. The results are representative of three similar experiments. $P<.05$ for the comparison between control and rapamycin for both RASM and human aortic smooth muscle cells at each time point after 48 hours.

Results

Rapamycin as low as 1 ng/mL, but not FK520 at any dose tested, inhibited RASM proliferation ($P<.05$, Fig 1). Rapamycin also decreased [^{3}H]thymidine incorporation in a dose-dependent manner ($P<.01$, Fig 2A). The inhibition of proliferation produced by rapamycin persisted at least through 7 days (168 hours) of cell culture. Similarly, rapamycin inhibited human aortic smooth muscle cell proliferation by 50% after 72 hours ($P<.05$; Fig 1, inset). In contrast, FK520 increased cell growth compared with control, but the differences were not significant. Cell viability, as assessed by trypan blue staining, was >99% in control, rapamycin-treated, and FK520-treated cells. The effect of rapamycin in terms of inhibiting DNA synthesis was competed by a molar excess of FK520 (Fig 2B). This result indicates that the reduction in [^{3}H]thymidine incorporation was probably mediated by rapamycin binding to the immunophilin FKBP, since both rapamycin and FK520 share this same cytosolic receptor. FK520 at low concentrations (eg, 2.5 ng/mL) caused a small but significant ($P<.05$) decrease in DNA synthesis (Fig 2A), and there was a small additive effect of FK520 (only at low concentrations, eg, 8 and 16 ng/mL) combined with rapamycin in terms of decreasing DNA synthesis (Fig 2B).

Rapamycin, but not FK520, delayed progression from G1 to S as assessed by cell-cycle analysis using propidium iodide staining. After stimulation with 20% FCS, ≈30% of cells progressed from G1/S and G2/M. The effect of rapamycin was to reduce progression from G1/S and G2/M to ≈10% of cells. pRb phosphorylation is believed to be a marker for progression from G1 to S. Hypophosphorylated pRb suppresses the progression from G1 to S,[26] and hyperphosphorylation generally occurs 1 to 2 hours before the G1/S transition.[27,28] Cells in early G1 contain exclusively hypophosphorylated pRb. At an undefined point in late G1, pRb is hyperphosphorylated and remains hyperphosphorylated until M. In quiescent RASM (maintained in 0.5% FCS for 72 hours), pRb phosphorylation occurred 6 to 8 hours after stimulation with 20% FCS. Culturing cells with rapamycin (100 ng/mL) delayed the onset of pRb hyperphosphorylation in RASM by 6 hours to ≈12 hours after G0 and reduced the levels of phosphorylation at each time point sampled (Fig 3). In contrast, FK520 nonsignificantly accelerated the time course of pRb phosphorylation (Fig 3).

Progression through the cell cycle is dependent on the activity of specific cell-cycle kinases, several of which, including cdk2 and cdk4, are thought to phosphorylate pRb. We examined the effects of rapamycin and FK520 on the activity of several cell-cycle kinases in VSMC. In RASM compared with control cells, p34^{cdc2} kinase activity was decreased ≈16 to 20 hours after G0 by rapamycin but not by FK520 (Fig 4A). Protein levels of p34^{cdc2} kinase were unchanged throughout the cell cycle (Fig 4A, insets). The decrease in p34^{cdc2} kinase activity at ≈16 to 20 hours corresponds to the G2/M transition in RASM. At earlier time points (during the G1/S transi-

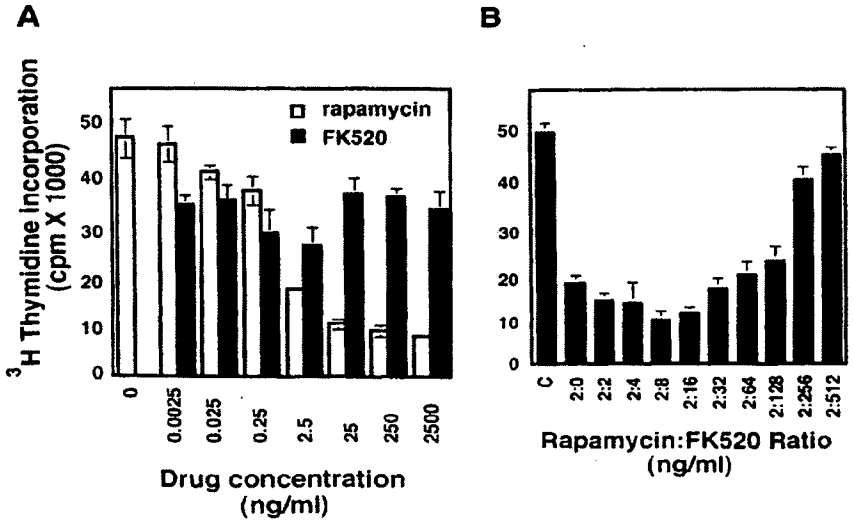

FIG 2. A, Bar graph showing the effect of immunosuppressive drugs rapamycin and FK520 on the incorporation of [^{3}H]thymidine in cultured rat aortic smooth muscle cells. B, Bar graph showing that FK520 competes with rapamycin for binding to FKBP12 and inhibits the effects of rapamycin on [^{3}H]thymidine uptake in cultured rat aortic smooth muscle cells. The results are a mean of quadruplicate wells, and the error bars represent standard deviation of the mean. $P<.05$ for the comparison between rapamycin-treated and control cells at each concentration above 0.0025 ng/mL. The results are representative of three similar experiments.

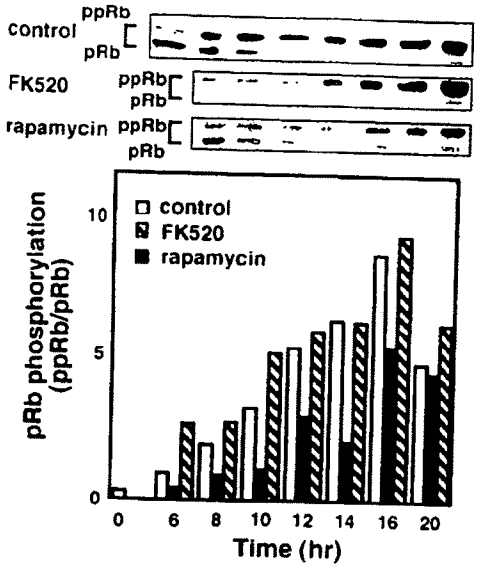

FIG 3. Bar graph showing the effects of rapamycin and FK520 on phosphorylation of retinoblastoma protein in cultured rat aortic smooth muscle cells. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. The indicated times are in hours after G0. The positions indicating hyperphosphorylation and underphosphorylation (ppRb and pRb, respectively) are indicated at the left of each gel panel (inset above graph). The results shown are from a representative experiment. Similar results were obtained in three experiments.

tion), $p34^{cdc2}$ kinase activity was low despite steady levels of $p34^{cdc2}$ protein, suggesting that it may have little effect at this point in the cell cycle in RASM (Fig 4A).

Compared with control cells, rapamycin (100 ng/mL) decreased $p33^{cdk2}$ kinase activity beginning at 10 hours through 16 hours after G0 (Fig 4B). The period from 10 to 16 hours after G0 corresponds to the time during which pRb phosphorylation is decreased by rapamycin-FKBP (Fig 3). Protein levels for $p33^{cdk2}$ kinase were unchanged throughout the cell cycle compared with control cells (Fig 4B, insets), indicating that the decrease in $p33^{cdk2}$ kinase activity was not due to a decrease in $p33^{cdk2}$ synthesis. These data suggest that the inhibition of pRb phosphorylation could at least in part be due to a decrease in $p33^{cdk2}$ kinase activity.

A regulatory role for cyclin D1 has been proposed with regard to pRb phosphorylation.[29,30] Interactions between cyclin D1 and a variety of cyclin-dependent kinases have been reported, and the expression of D-type cyclins is regulated by growth factors.[31] We sought to determine, on the basis of these observations, whether the antiproliferative effects of rapamycin in RASM were associated with regulation of cyclin D1. Cyclin D1 levels were elevated in control RASM at ≈10 hours after G0, corresponding to the onset of pRb phosphorylation. Rapamycin delayed the onset of this rise in cyclin D1 levels by 4 to 6 hours (data not shown). The reduction in cyclin D1 levels by rapamycin occurred at the point when pRb phosphorylation was reduced.

Discussion

Our data show that the antiproliferative effects of rapamycin in VSMC are associated with an inhibition of cell-cycle kinases, cyclins, and pRb phosphorylation. These data imply that phosphorylation of pRb plays an important role in signaling during smooth muscle proliferation. In contrast, FK520, another potentially useful drug for immunosuppression following cardiac transplantation, induces a nonsignificant increase in VSMC proliferation associated with an acceleration of the time

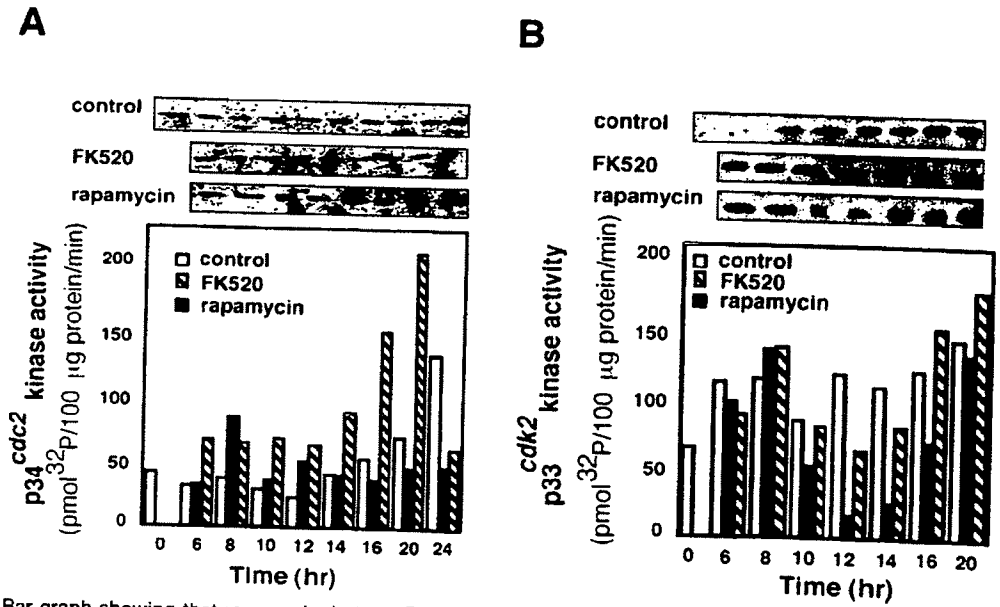

FIG 4. A, Bar graph showing that rapamycin, but not FK520, decreases $p34^{cdc2}$ kinase activity at the G2/M transition in cultured rat aortic smooth muscle cells. The insets are immunoblots showing $p34^{cdc2}$ protein levels. Cells were treated with either no drug, 100 ng/mL rapamycin, or 100 ng/mL FK520. B. Bar graph showing that rapamycin, but not FK520. decreases $p33^{cdk2}$ kinase activity at the G1/S transition. The insets are immunoblots showing $p33^{cdk2}$ protein levels. With the exception of the earliest two time points in the control samples, the level of $p33^{cdk2}$ protein remained constant throughout the cell cycle. The results shown are from representative experiments. Similar results were obtained in three experiments.

course and extent of pRb phosphorylation (Figs 1 and 3). The antiproliferative effects of rapamycin appear to be mediated by binding to the cytosolic receptor FKBP because they are competed by FK520, a drug that shares the same receptor. However, these studies do not exclude the possibility that these drugs also interact with other binding sites in RASM.

We observed small but significant effects only at low concentrations of FK520 (2 to 20 ng/mL) in terms of inhibiting DNA synthesis. However, the physiological importance of these effects was questionable because we never observed any inhibition of RASM proliferation when using either FK520 or FK506 at any concentration. Indeed, to the contrary, we have consistently observed a small nonsignificant increase in proliferation when using either FK520 or FK506. Moreover, FK520 increases pRb phosphorylation and cell-cycle kinase activity (Figs 3 and 4), suggesting that it accelerates cell-cycle progression.

Cyclin-dependent kinases, including cdk2, have been implicated as regulators of pRb function.[32-34] Although the data suggest that a cyclin-dependent kinase phosphorylates pRb in vitro, there remains some controversy as to which kinase and how many actually carry out this function in vivo. We found that rapamycin but not FK520 decreased the activity of $p33^{cdk2}$ kinase[35] in VSMC. This finding does not indicate that pRb phosphorylation is dependent on $p33^{cdk2}$ kinase but suggests that this kinase could be involved in regulating pRb function in VSMC. We did not examine the activities of other kinases that similarly could be playing a role in phosphorylating pRb. Indeed, determining the specific kinase(s) that phosphorylates pRb would be interesting but is not required to support the main point of the present study, which is that the antiproliferative effects of rapamycin in VSMC are associated with inhibition of regulators of cell-cycle progression. Similarly, the finding that rapamycin decreased the activity of cell-cycle kinases and the levels of a cyclin (D1) does not rule out the possibility that rapamycin could have effects on other regulators of cell growth as well.

$p34^{cdc2}$ kinase is thought to play an important regulatory role in the G2/M transition.[36,37] The time course for inhibition of $p34^{cdc2}$ kinase activity by rapamycin suggests that this kinase may play an important role in the G2/M transition in VSMC. In another myogenic cell line, BC3H1 cells, we had previously shown that rapamycin inhibited proliferation and induced differentiation and that these effects were also associated with a reduction in $p34^{cdc2}$ kinase activity.[24] However, in BC3H1 cells, the decrease in $p34^{cdc2}$ kinase activity occurs at the G1/S transition. The $p34^{cdc2}$ kinase may have multiple roles in the cell cycle, depending on which cell type is examined.

The growth-inhibitory effects of rapamycin (Fig 1) in VSMC are long lasting. In contrast, inhibition of pRb phosphorylation (Fig 3) and cell-cycle kinase activity (Fig 4) appears to be more of a transient delay rather than a complete block. Moreover, examination of the cell growth data in Fig 1 shows that although growth is significantly suppressed by rapamycin, there is some slow growth in the rapamycin-treated cells. Indeed, taken together, these data suggest that rapamycin significantly lengthens the cell cycle by introducing delays at the G1/S and G2/M transition points. These delays appear to result in a marked prolongation of the doubling time for the VSMC exposed to rapamycin (Fig 1). Some of the cell-cycle kinase activity and phosphorylation of pRb observed later in the cell cycle (eg, at 16 to 20 hours) in the rapamycin-treated cells may reflect the fact that the cell cultures were not synchronized. Thus, a subset of cells was past the G1/S transition at the start of the experiment, despite culturing in low-serum medium for 72 hours to induce quiescence.

It is believed that immunologic events linked to HLA incompatibility between the donor and host may result in vascular injury, leading to VSMC proliferation. Moreover, accelerated arteriosclerosis is not limited to cardiac transplant patients, as other organ allografts are subject to similar processes.[38] Cyclosporin A, one of the most widely used immunosuppressants, appears to have a neutral effect on accelerated arteriosclerosis.[6,15,38,39] The mechanisms underlying post–cardiac transplant–accelerated arteriosclerosis remain poorly understood.[5,15,38,40] Nevertheless, VSMC proliferation is the fundamental pathology. Accelerated arteriosclerosis after cardiac transplantation occurs with similar frequency despite the use of newer immunosuppressant agents, including cyclosporin A and FK506. FK506 is currently being used as a therapeutic agent for the prevention of post–cardiac transplant rejection in humans. Our findings predict that FK506 would either be neutral in terms of VSMC proliferation or could have an adverse effect by accelerating the time course and the extent of posttransplant arteriosclerosis. Conversely, since rapamycin both immunosuppresses and blocks VSMC proliferation, it could be the preferred therapeutic agent to reduce accelerated arteriosclerosis following cardiac transplantation and might even prolong survival in cardiac transplant recipients.

Many studies have attempted to identify the factor or factors contributing to VSMC proliferation following vascular injury, particularly after PTCA.[2,9,12,21,22,41-46] The fact that rapamycin inhibits cell-cycle dependent kinases and phosphorylation of pRb suggests that its effects on VSMC proliferation would not depend on which of the many agents capable of triggering VSMC proliferation after injury are causative. As such, rapamycin might also be a useful agent for reducing or blocking the component of post-PTCA restenosis that is due to VSMC proliferation.

Acknowledgments

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CONTENTS

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Editorial Note

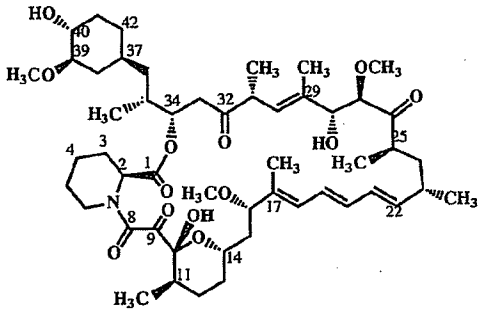
The authors of the article "New triene-β-lactone antibiotics, triedimycins A and B", Y. IKEDA *et al.* in Journal of Antibiotics 44: 453~455, 1991, have communicated to the editor as follows;

"Triedimycins A and B were found to be identical with curromycins A and B, respectively, in their properties and structures (M. OGURA *et al.*: J. Antibiotics 38: 669~673, 1985 and Agric. Biol. Chem. 49: 1908~1910, 1985). We would suggest that the name of curromycins A and B should be used for these antibiotics to avoid confusion."

Correction

Communication to the Editor by J. B. MCALPINE, S. J. SWANSON, M. JACKSON & D. N. WHITTERN: Revised NMR assignments for rapamycin. J. Antibiotics 44, No. 6, pp. 688~690, June 1991

p. 688 right structure should be replaced by the following:



Rapamycin

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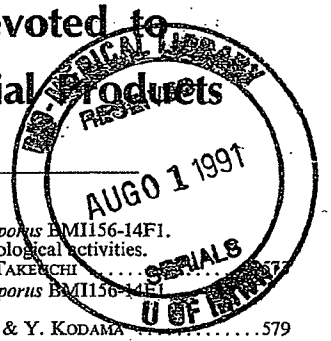

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REVISED NMR ASSIGNMENTS FOR RAPAMYCIN

Sir:

Recently, considerable interest has been given to the antifungal antibiotic, rapamycin,[1,2] because of its immunosuppressant properties[3] and its clear structural relationship to FK-506. FK-506 and rapamycin strongly inhibit the peptidyl-prolyl isomerase, FK-506-binding protein,[4] and this may be the crux of the mechanism of their immunosuppressive properties. Despite the strong similarity in the structures of the presumed pharmacophores of these two entities, differences exist in their biological effects. Whereas FK-506 inhibits production of IL-2 and the IL-2 receptor, rapamycin appears to inhibit neither.[5]

The structure of rapamycin was deduced by X-ray analysis[6] and a detailed NMR analysis has been published.[7] In $CDCl_3$, rapamycin exists as a mixture of conformers in a ratio of about 3 : 1. This further complicates already complex spectra, particularly the proton spectrum. An investigation of proton and carbon spectra of rapamycin indicated that many of the assignments made in 1980 (without the benefit of the modern 2D methods) were incorrect. With the aid of COSY, HETCOR and DEPT experiments we have reassigned the proton and carbon spectra from basic principles. The results are presented in Table 1.

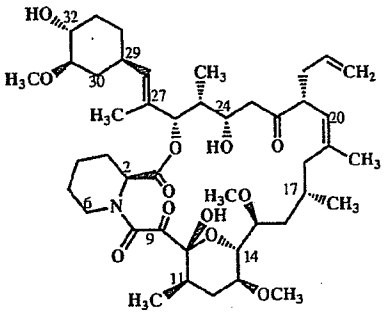
The numbering system used here is that commonly used for macrolides and which has been used for FK-506.[8] It is different from that used previously for rapamycin but the authors believe it is preferable. Assignments listed in Table 1 take account of the difference in numbering systems. Although many of the reassignments, especially in the carbon spectra, are trite, for example, the interchange in the assignment of peaks at 27.0 and 27.3 ppm to C-3 and C-12, others are of considerable significance such as the resonances assigned to C-19, C-20, C-22 and C-30 and the *C*- and *O*-methyl carbons.

These data will be of considerable significance to future work on the biosynthesis and derivatization of rapamycin.

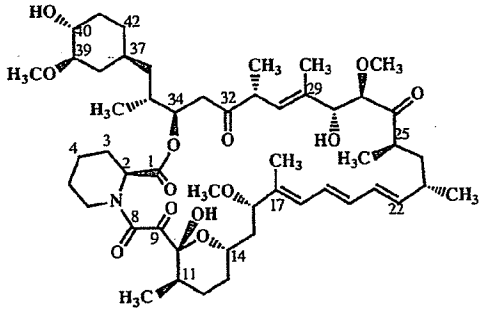
Experimental

Rapamycin was produced by fermentation of *Streptomyces hygroscopicus* ATCC 29253 using the media and conditions previously described.[9] Antibiotic was isolated from the fermentation broth by addition of polystyrene resin, Amberlite XAD-16, at 10%. The mixture was stirred for 2 hours, centrifuged and decanted to obtain a mixed paste of resin and mycelia. This was extracted twice with two volumes of acetone. The combined acetone extracts were concentrated under reduced pressure to an aqueous slurry which was further extracted twice with toluene. Emulsions were broken by the addition of acetone (*ca.* 5%) to the mixture. The toluene layer was concentrated and the oily residue was chromatographed over silica gel developed with a gradient from hexane and acetone 1 : 1 to 100% acetone. Rapamycin-containing fractions were pooled, concentrated and rechromatographed over Sephadex LH-20 in methanol. Fractions containing rapamycin were combined and rechromatographed on Sephadex LH-20 in chloroform - heptane - ethanol (10 : 10 : 1). Fractions containing only rapamycin, as analysed by TLC (Merck Kieselgel 60 HF_{254} developed in hexane - acetone, 1 : 1) were combined and concentrated to a solid residue which was used for these NMR experiments.

NMR experiments were carried out on a General Electric GN500 spectrometer in $CDCl_3$ with TMS



FK-506



Rapamycin

Table 1. Reassigned chemical shifts for the carbons and protons of rapamycin.

Carbon No.	Carbon type	Major carbon	Minor carbon	Major proton	Minor proton	Previous major carbon	Assignments[7] major proton
1	C=O	169.2				169.2	
2	CH	51.3	56.2	5.29	4.29	51.4	5.26
3	CH_2	27.0		2.34, 1.76	?, 1.84	27.3	1.92, 1.60
4	CH_2	20.6		1.78, 1.47		20.6	
5	CH_2	25.3		1.75, 1.48		25.3	
6	CH_2	44.2	39.8	3.59, 3.44	4.44, 3.2	44.2	3.56, 3.40
8	C=O	166.8				166.8	
9	C=O	192.5				192.5	
10	O–C–OH	98.5	98.8			98.5	
11	CH	33.7		1.98		33.2	1.95
12	CH_2	27.3		1.60, 1.60		27.0	2.32, 1.45
13	CH_2	31.3		1.62, 1.33	1.53, ?	38.4	
14	CH–OC	67.2	67.8	3.86	3.79	67.2	3.85
15	CH_2	38.8	38.8	1.85, 1.52	1.87, 1.58	38.8	1.73
16	CH–OCH_3	84.4	84.4	3.67	3.61	84.4	3.64
17	C=C	135.5				135.5	
18	CH=C	129.6	129.2	5.97	5.89	129.6	5.94
19	CH=C	126.4		6.39		133.6	6.28
20	CH=C	133.6	133.6	6.32	6.25	126.4	6.36
21	CH=C	130.1	129.9	6.15	6.15	130.2	6.12
22	CH=C	140.2	140.9	5.54	5.51	126.7	5.51
23	CH	35.2	35.6	2.32	2.30	35.2	2.29
24	CH_2	40.2	40.2	1.50, 1.20	1.47, 1.20	31.6	1.83, 1.68
25	CH	41.4	41.0	2.74	2.87	41.4	2.72
26	C=O	215.6				215.5	
27	CH–OCH_3	84.9	86.3	3.71	3.67	84.9	3.70
28	CH–OH	77.3	77.2	4.17	4.20	77.2	4.15
29	C=C	136.1				136.1	
30	CH=C	126.8	126.8	5.42	5.49	140.1	5.38
31	CH	46.6		3.33		46.6	3.30
32	C=O	208.2				208.1	
33	CH_2	40.7	41.0	2.74, 2.60	2.87, 2.74	40.7	2.72, 2.56
34	CH–OCO	75.7		5.17	5.12	75.7	5.15
35	CH	33.1		1.98	2.01	33.7	
36	CH_2	38.4		1.22, 1.12		40.2	
37	CH	33.2		1.39	1.38	33.2	1.50
38	CH_2	34.2	34.2	2.10, 0.68	2.09, 0.64	34.2	2.08, 0.64
39	CH–OCH_3	84.4	84.4	2.93	2.90	84.4	2.91
40	CH–OH	73.9	73.9	3.37	3.35	73.9	3.35
41	CH_2	31.3		1.99, 1.33	1.97, 1.30	31.3	
42	CH_2	31.7		1.70, 1.00		31.3	
43	11-CH_3	16.2		0.95		13.8	0.92
44	17-CH_3	10.2		1.65	1.74	13.0	1.62
45	23-CH_3	21.5		1.05		15.9	1.02
46	25-CH_3	13.8		1.00		21.5	0.96
47	29-CH_3	13.0		1.74	1.65	10.1	1.72
48	31-CH_3	16.0		1.11		15.9	1.06
49	35-CH_3	15.9		0.92		16.2	0.89
50	16-OCH_3	55.8		3.13		59.4	3.32
51	27-OCH_3	59.5		3.34	3.40	56.5	3.39
52	39-OCH_3	56.5		3.41	3.36	55.8	3.11

as external reference. ^{1}H NMR spectra, COSY, HETCOR and LRHETCOR were measured at 499.96 MHz. ^{13}C NMR spectra and DEPT experiments were performed at 125.64 MHz.

Addendum in Proof

After this note had been accepted, a paper on the biosynthesis of rapamycin appeared. (N. L. PAIVA, A. L. DEMAIN and M. F. ROBERTS: J. Nat. Products. 54: 167~177, 1991) It included several reassignments for the ^{13}C NMR signals of the major rotamer of rapamycin. We agree with all of the reassignments made but our results indicate that seven more carbons still require revision. These include interchanges between the three methoxyl carbons, and interchanges between the pairs of carbons 11 and 35, 3 and 12. This last interchange is particularly significant to the interpretation of the results of the biosynthetic experiments. The authors had difficulty in finding a precursor to carbons C-12, C-13, postulated as part of a polyketide chain. These new assignments indicate that 1-[^{13}C]-acetate led to significant enrichment (to 2.4%) in C-12. Enrichment into C-13 is more difficult to ascertain because signals for this carbon and C-41 are coincident in this solvent.

Acknowledgments

We are grateful to Professor A. DEMAIN, M. I. T. for an authentic sample of rapamycin.

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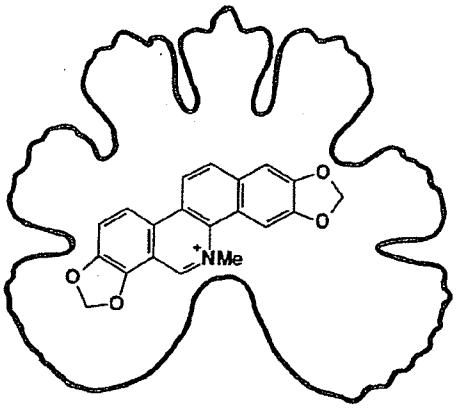
JOURNAL OF NATURAL PRODUCTS



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INCORPORATION OF ACETATE, PROPIONATE, AND METHIONINE INTO RAPAMYCIN BY *STREPTOMYCES HYGROSCOPICUS*

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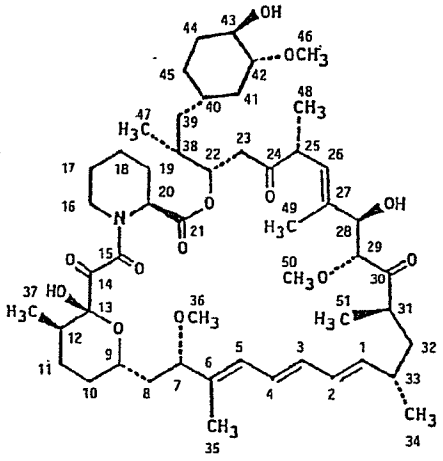
ABSTRACT.—Labelling experiments with ^{13}C-labelled precursors demonstrated that the majority of the macrolide ring of rapamycin [**1**] is formed from six acetate and seven propionate units. A two-carbon unit in the ring was not labelled by acetate, propionate, glycine, or methionine. Methionine and glycine did, however, label the three methoxy groups of rapamycin to a high degree. Results from the incorporation experiments demonstrated that there were errors in the published ^{13}C-nmr spectral assignments for rapamycin; these have now been corrected.

Rapamycin [**1**] was first reported as an antifungal agent in 1975 by Vezina *et al.* (1). It is produced by a strain of *Streptomyces hygroscopicus* isolated from an Easter Island soil sample. It is very active against *Candida* species (common human pathogenic yeasts) and moderately active against dermatophytes, while having no activity against bacteria (2). Its specificity, relatively low toxicity, and good oral absorption in mice and dogs (3) make rapamycin a potentially valuable antifungal agent. Rapamycin was also shown to have good activity against mammary, colon, and brain tumor model systems (4) and against transplanted tumors (5). It inhibits the immune response in rats, apparently by inhibiting the lymphatic system (6). The antifungal mode of action of rapamycin is still unclear, although it appears to differ from those of many known antifungal agents (7,8).



1

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The structure of rapamycin [1] was determined by a combination of X-ray crystallography and ^{13}C- and ^{1}H-nmr experiments (9,10). A major portion of the molecule is a very large (31-membered) "macrolide ring." Three conjugated double bonds (C-1 to C-6) allow rapamycin to be classified as a "triene," an uncommon type of polyene. A rare α-ketoamide functionality (C-14 and C-15) is located just outside a hemiketal ring, resulting in a series of three highly oxidized carbons. C-15 forms an amide bond with the sole nitrogen atom present in the antibiotic. The heterocyclic ring including this nitrogen and C-16 to C-20 plus the C-21 carboxyl can be excised by acid- or base-catalyzed hydrolysis as L-pipecolic acid (10). Outside the macrolide ring is a trisubstituted cyclohexane ring (C-40 to C-45). Many antibiotics contain C_6 rings, but they are usually aromatic. A few other antibiotics contain cyclohexane rings, such as asukamycin and manumycin, but little has been published about their biosynthesis (11,12). At the time of its discovery, many structural features of rapamycin were unique. Recently, FK506, a promising immunosuppressant from *Streptomyces tsukubaensis*, was found to have many structural features identical to rapamycin (13). Information learned about the biosynthesis of rapamycin may also apply to the same moieties of FK506.

Little was known about the biosynthesis of rapamycin when this work was begun. Based on what was known about the biosynthesis of macrolides, ansamycins, and other similar natural products, we proposed a pathway for rapamycin biosynthesis (Figure 1). We hypothesized that a cyclic C_7 unit derived from the shikimic acid pathway would serve as the starter unit. The long polyketide chain (macrolide ring) would then be built up from the condensation of seven acetate and seven propionate units. Chain growth

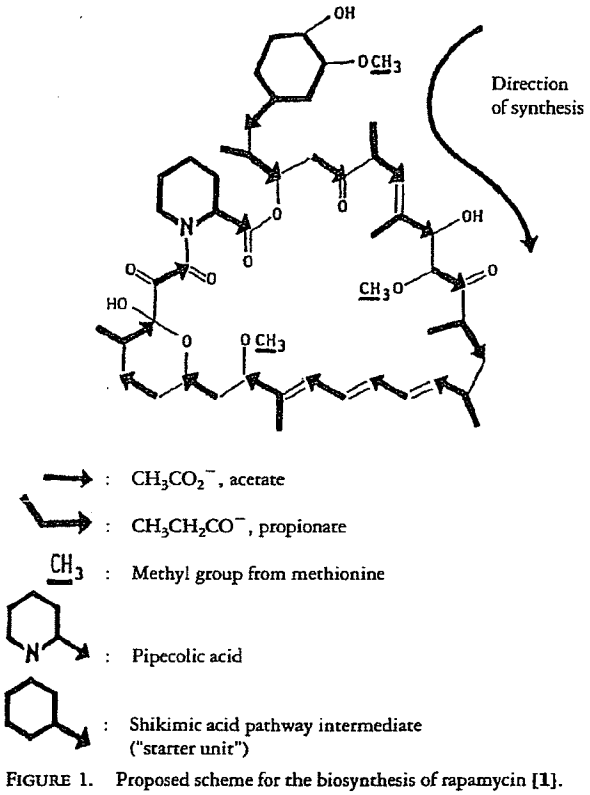


FIGURE 1. Proposed scheme for the biosynthesis of rapamycin [1].

would be terminated by the attachment of the pipecolate moiety or by lysine, which would later be cyclized to form the pipecolate moiety. The pipecolate carboxy group and the C-22 hydroxyl would then interact to close the ring by lactone formation. Some additional oxidations and reductions would occur, and three hydroxyl groups would be methylated, presumably with *S*-adenosylmethionine (SAM) acting as the methyl donor.

In this paper we report the results of experiments designed to test parts of the above proposed pathway and to determine the biosynthetic origins of a large number of the carbons of rapamycin.

EXPERIMENTAL

STRAINS.—*S. hygroscopicus* strain AY-B1206 was obtained from the Ayerst Research Laboratories, Montreal, Canada. This strain is a soil isolate found during a re-examination of the soil sample which contained the original rapamycin-producing strain (AYB994, deposited as ATCC 29253 and NRRL 5491). It was reported (personal communication from S.N. Sehgal) to produce higher levels of rapamycin and little or no demethoxyrapamycin when compared to the original isolate.

MEDIA.—*Sporulation agar.*—One liter of agar contains 20 g glucose, 1 g KNO_3, 0.5 g K_2HPO_4, 0.1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 22 g agar (pH 6.8). The agar was melted and distributed into square bottles; after autoclaving, the bottles were placed on their sides to provide a thin layer of agar.

Fermentation medium for labelling studies.—The following were added to 900 ml distilled H_2O: 2.0 g K_2HPO_4, 2.0 g KH_2PO_4, 5.0 g NaCl, 1.5 g $CaCO_3$, 20 ml glycerol, 1.0 g L-leucine, 2.0 g L-glutamic acid, 4.0 g L-lysine·HCl, 5.0 g yeast extract (Difco), 0.06 g $ZnSO_4$, 0.256 g $MgSO_4 \cdot 7H_2O$, 0.012 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.018 g $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.01 g $Na_2B_4O_7 \cdot 10H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.0013 $CuCl_2 \cdot 2H_2O$, 0.51 g $MgCl_2 \cdot 6H_2O$, and 0.36 g $NaSO_4$. The pH of the solution was adjusted to 6.0. This solution (45 ml) was placed into 500-ml unbaffled Erlenmeyer flasks. The flasks were autoclaved for 20 min at 121°. After cooling, 5.0 ml of sterile 20% glucose was added to each flask.

CULTURE CONDITIONS.—Fermentation flasks were inoculated directly with a spore suspension. To prepare the inoculum for fermentations, several bottles of sporulation agar were inoculated with spore stock. Bottles were incubated for 14–21 days at 25°. During this time, the culture sporulated, changing from white to dark grey. A spore suspension was prepared by adding sterile H_2O to the bottles and suspending the spores by scraping the agar surface with an inoculating loop or spatula. Spores from 200 to 250 cm^2 of agar were suspended in approximately 15 ml H_2O. For labelling work, one ml of the suspension was used to inoculate 50 ml of fermentation medium in 500-ml unbaffled flasks. Fermention flasks were shaken at 25° (2″ diameter, 250 rpm) until the pH reached 7.0, usually 6 to 7 days. During the course of the fermentation, 1-ml samples of broth were removed from at least duplicate flasks. The pH of each sample was measured. Samples were assayed for rapamycin as described below. The final concentration of rapamycin was 50 to 60 mg/liter.

To determine the dry cell weight of the cultures, 2 ml of 3 N HCl was added to each 50-ml flask to dissolve any remaining $CaCO_3$. The complete contents of a flask were then collected by filtration, dried at 90° for 24 h, and weighed.

HPLC ASSAY FOR RAPAMYCIN.—MeOH (9 ml) was added to each 1-ml sample of fermentation broth, and the tubes were tightly sealed and shaken several times during 20 min to extract the rapamycin. The tubes were centrifuged and the supernatant fluids filtered through glass fiber filters or solvent-resistant 0.45-μm filters. These samples were either analyzed immediately or stored at −20° until analysis. The rapamycin content was determined by reversed-phase hplc (Waters μBondapak C-18 column, 4 mm × 30 cm; mobile phase 70% MeOH/30% H_2O, 2 ml/min; detected at 254 nm and 280 nm, Waters Model 440 UV and Waters Lambda-Max Model 480 absorbance detectors at 0.01 aufs); 50 μl of extract was injected. Rapamycin has a retention time of approximately 30 min.

Purified rapamycin (a gift of Ayerst Laboratories) was dissolved in MeOH and used to prepare a series of standard solutions. The concentration of rapamycin was found to be directly proportional to peak height. A 10 μg/ml standard solution gave a peak of approximately 70% of the full scale.

SMALL-SCALE RECOVERY AND PURIFICATION OF RAPAMYCIN.—To prepare rapamycin samples for nmr and tlc analysis, the contents of several fermention flasks were combined. The mycelium was collected by centrifugation for 10 min (4000 *g*, GSA rotor) at room temperature. The pellet was resuspended in distilled H_2O and centrifuged again. The pellet was then resuspended in approximately 4 times its volume of MeOH. This mixture was shaken to extract the rapamycin and then centrifuged for 10 min. The superna-

tant (MeOH) was removed, and the extraction repeated two more times with similar volumes of fresh MeOH. The extracts were combined and the MeOH removed by rotary evaporation. The resulting oil was dissolved in CH_2Cl_2 (150 ml per liter of broth being processed) to which an equal volume of H_2O was added. A dark emulsion layer often appeared at the interface. The CH_2Cl_2 layer was removed, and the oil/emulsion was extracted 2 more times with similar volumes of CH_2Cl_2. The CH_2Cl_2 extracts were pooled and the solution evaporated to dryness. The resulting residue was extracted with several portions of hexane. The hexane was removed by rotary evaporation, leaving a dark yellowish oil. At this stage, the sample was redissolved in a known volume of hexane and a portion applied to tlc plates (as in the experiments for determining ^{14}C incorporation). Alternatively, the sample was dissolved in approximately 2 ml MeOH and further purified by semipreparative-scale hplc (Waters μBondapak C18, 8 mm × 30 cm; 254 and 280 nm UV detectors; typically 65% MeOH/35% H_2O, 5 to 7 ml/min). The center portion of the rapamycin peak was collected.

The collected fractions were pooled and concentrated by one of two procedures. For most of the samples, the fractions were chilled in an Me_2CO/dry ice bath, and the MeOH/H_2O evaporated under vacuum in a lyophilizer. For two samples (2-^{13}-C-propionate and $^{13}C_2$-glycine experiments), C-18 reversed-phase cartridges (Maxi-Clean Cartridges, Alltech, Deerfield, Illinois) were used. Sufficient H_2O was added to the pooled fractions to bring their concentration to approximately 40% MeOH, and the solution was passed through the cartridge to bind the rapamycin to the C-18 packing. Air was passed through the cartridge briefly to remove as much solvent as possible, and the rapamycin was eluted with pure MeOH. The MeOH was removed by rotary evaporation without heating, and any remaining traces of H_2O were removed by lyophilization. After concentration, the rapamycin was collected by dissolving in CH_2Cl_2, the solution filtered and transferred to a small flask, and the CH_2Cl_2 removed by rotary evaporation. The sample was stored at −20° until analyzed by ^{13}C nmr.

^{13}C-NMR SPECTROSCOPY.—Two different spectrometers were used for acquiring ^{13}C-nmr spectra of rapamycin in $CDCl_3$ (unless otherwise noted) in 10 mm tubes: a Bruker 270 Spectrometer (67.9 MHz) and a home-built wide-bore 360 (90.4 MHz). At 67.9 MHz acquisition parameters included a 16 kHz sweep width, 16K data points, 40° pulse width (12–16 μsec), and a relaxation delay of 1.5 sec. ^1H noise-decoupled spectra were recorded at ambient probe temperature (30–33°). The number of transients accumulated ranged from 14,000 to 16,000 for the natural abundance sample (100 mg) and 8000 (for highly enriched) to 17,000 for the enriched samples (8 to 12 mg). To enhance sensitivity, each free induction decay was treated with a 4-Hz line broadening. For acquisition of ^{13}C spectra at 90.4 MHz the following parameters were used: 20 kHz sweep width, 16K data points, 90° pulse width (18.5 μsec), and 5 sec relaxation delay. Protons were decoupled by a WALTZ sequence. Probe temperature was 22° in this spectrometer. The number of transients ranged from 4000 to 5000 for natural abundance (100 mg) and 5000–6000 for ^{13}C-enriched (approximately 10 mg) samples. To enhance sensitivity, a 2-Hz line broadening was applied to free induction decays.

RESULTS

GROWTH, PRODUCTION, AND pH.—A typical fermentation profile is shown in Figure 2. Because rapamycin accumulation was first detectable between 2 and 2.5 days, this was the time chosen for the addition of labelled precursors. Rapamycin is unstable at alkaline pH; cultures were harvested before pH 7 was reached.

INCORPORATION OF LABELLED ACETATE.—Initial efforts were focused on the origins of the polyketide chain, beginning with the simplest available precursor, acetate. Preliminary experiments showed that the maximum amount of sodium acetate that could be added to the culture at 2.5 days was 0.5 g/liter; higher concentrations caused decreased rapamycin production and cell death. Experiments with 1-[^{14}C]- plus 2-[^{14}C]-acetate indicated that this concentration of acetate should provide suifficient, although low, enrichment of rapamycin in the corresponding ^{13}C experiment. In separate experiments, 1-[^{13}C]- and 2-[^{13}C]-acetate were added to cultures, and the labelled rapamycin was recovered and analyzed. We obtained 7% total incorporation of 1-[^{14}C]- and 2-[^{14}C]-acetate or an estimated 1% at each of seven sites. The relative ^{13}C enrichments are shown in Table 1. The maximum uncertainty in these values is 25%. Due to the small sample size, the signal-to-noise ratios were relatively low. However, careful comparison of the spectra revealed that several carbons were significantly labelled. C-5, C-9, C-15, C-24, and C-30 were enriched by 1-^{13}C-acetate as predicted by the pro-

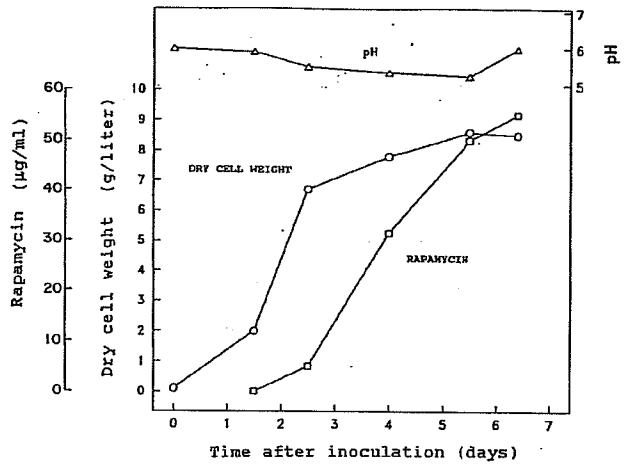


FIGURE 2. Rapamycin production, growth, and pH during fermentation.

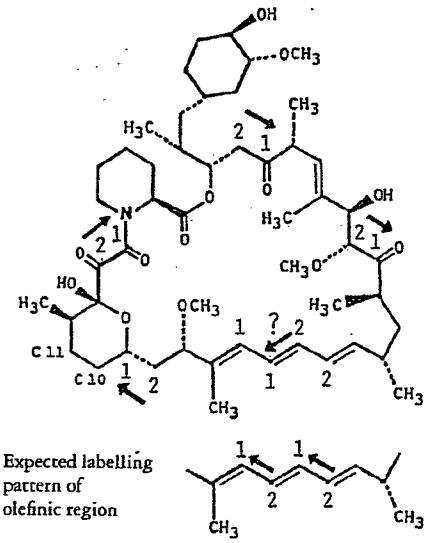
posed scheme. Surprisingly, C-3 and C-11 (predicted to be derived from C-1 of acetate) were not significantly enriched, while C-4 (predicted to be derived from C-2 of acetate) was highly enriched. For the sample prepared with 2-^{13}C-acetate, similar results were obtained. C-2, C-8, C-14, C-23, and C-29 were enriched by 2-^{13}C-acetate as predicted, while C-10 and C-4 (predicted to be labelled) were not, and C-3 (predicted to be derived from C-1 of acetate) was definitely enriched. In addition, in both samples, several carbons thought to be derived from propionate were also apparently enriched by labelled acetate. The latter enrichment of propionate carbons by acetate could be attributed to metabolic scrambling of the labelled acetate carbons into propionate/methylmalonyl-CoA via the citric acid cycle. The lack of significant incorporation at C-10 and C-11 suggested that these two carbons are not derived from acetate. The enrichment of the resonance assigned to C-3 by 2-[^{13}C]-acetate and that assigned to C-4 by 1-[^{13}C]-acetate was most surprising; it implied that a strange rearrangment involving C-C bond breakage and formation might have occurred. An alternative explanation was that an error had been made in the ^{13}C-nmr assignments of C-3 and C-4 (10). Eight of the carbons that had been labelled as predicted (C-23, C-24, C-29, C-30, C-8, C-9, C-14, and C-15) correspond to four of the predicted seven acetate units, in the predicted orientation (Figure 3).

In order to determine if any error had been made in the published ^{13}C-nmr spectral assignments (10) and to confirm the above conclusions, doubly labelled acetate (1,2-[$^{13}C_2$]-acetate) was incorporated into rapamycin using the same procedures as for the singly labelled samples. If the acetate is incorporated intact, the nuclei of the two adjacent ^{13}C atoms will still exhibit ^{13}C-^{13}C coupling, resulting in the appearance of a satellite doublet ("coupling pattern") flanking the singlets corresponding to the two rapamycin carbons enriched. If the acetate C-C bond is broken and the two ^{13}C atoms are separated by scrambling reactions, subsequent incorporation will increase the intensity of the singlet resonance without the appearance of the ^{13}C-^{13}C doublet. As indicated in Table 1, a total of 12 "coupled" carbons (J_{CC} = 40 to 60 Hz) were identified after incorporation of 1,2-[$^{13}C_2$]-acetate into rapamycin, indicating the incorporation of six intact acetate units. Four of these are the four acetate units labelled as predicted by singly labelled acetate. The other two correspond to C-2 through C-5, implying the incorporation of two intact acetate units into the olefinic region. This contradicts the re-

TABLE 1. Relative ^{13}C-Enrichment Factors.[a]

Chemical shift (ppm)	Published[b] carbon assignment	1-[^{13}C]-acetate	2-[^{13}C]-acetate	1,2-[$^{13}C_2$]-acetate		1-[^{13}C]-propionate	2-[^{13}C]-propionate	Reassigned[d] carbon no.
				uncoupled	coupled[c]			
215.5	30	3.0[e]	1.0	1.5	yes			
208.1	24	3.9[e]	1.2	1.5	yes			
192.5	14	0.9	2.3[e]	1.5	yes			
169.2	21	1.0	0.9	1.5				
166.8	15	2.4[e]	0.8	1.4	yes			
140.1	26	2.0[f]	1.2	2.1		3.0[e]		1
136.1	27	1.3	1.9[f]	2.2			3.0[e]	
135.5	6	1.4	1.7[f]	2.4			2.5[e]	
133.6	4	3.5[g]	0.8[g]	1.5	yes			3
130.2	2	1.3	2.4[e]	1.7	yes			A[h]
129.6	5	3.0[e]	0.9	1.7	yes			A
126.7	1	1.9[f]	2.1[f]	2.6		3.0[e]		26
126.4	3	1.1[g]	3.3[g]	1.6	yes			4
98.5	13	2.4[f]	1.3	2.3		2.0[e]		
84.9	29	1.3	2.2[e]	1.4	yes			
84.4	7	1.6[f]	1.4	1.9		2.5[e]		
84.4	42							
77.2	28	(obscured by solvent peaks)				2.5[e]		
75.7	22	1.9[f]	0.7	—		2.7[e]		
73.9	43	1.2	1.2	1.0				
67.2	9	3.1[e]	1.1	1.7	yes			
59.4	36	1.0	0.9	1.0				
56.5	50	1.0	1.1	0.9				
55.8	46	0.9	1.1	1.1				
51.4	20	1.1	0.8	1.2				
46.5	25	1.3	2.1[f]	2.3			3.0[e]	
44.2	16	1.3	0.9	1.2				A
41.4	31	1.4	2.0[f]	2.7			2.2[e]	
40.7	23	1.1	2.4[e]	1.7	yes			A
40.2	39	1.4	1.3	2.0		2.1[g]		32
38.9	8	0.8	1.7[e]	1.6	yes			A
38.4	10	1.1	0.7[g]	1.1	no[g]			39
35.2	33	1.1	2.8[f]	2.6			2.1[e]	
34.2	41	0.9	2.6[e]	1.2				A
33.7	38	1.1	1.9[f]	2.2			2.0[e]	
33.2	40	0.9	1.5	1.6				
33.2	12						2.0[e]	
31.7	32	0.7	0.8	1.0		g		45
31.3	45	0.8	1.4	1.3				10
31.3	44							A
27.3	19	2.4[f]	1.6[f]	1.8				A
27.0	11	1.4[g]	0.9	1.7	no[g]			A
25.3	17	1.0	0.7	1.2				A
21.5	48	0.7	1.7[f]	1.6				34
20.6	18	0.8	0.8	0.9				A
16.2	47	0.6	1.3	1.8				37
15.9	51	0.8	1.3	1.9				47
15.9	34							48
13.8	37	0.6	1.4	1.7				51
13.0	35	0.9	1.8[f]	2.1				49
10.1	49	0.8	1.2	1.6				35

[a]Natural abundance = 1.0. Reference: average of three methoxy carbon signals.
[b]These assignments are from Findlay and Radics (10).
[c]The average coupling constants (J_{cc}) after peak reassignments for the six pairs of carbons were $J_{2,3}=53$, $J_{4,5}=58$, $J_{8,9}=41$, $J_{14,15}=61$, $J_{23,24}=39$, $J_{29,30}=41$ Hz.
[d]Dr. R.P. Borris and Dr. B.H. Arison, personal communication.
[e]Significantly enriched, as predicted by proposed scheme.
[f]Enrichment attributed to incorporation after metabolic scrambling.
[g]Not as predicted; possible nmr misassignment.
[h]Indicates assignments agree with those of Findlay and Radics (10).



1 : Enriched by 1-[^{13}C]-acetate

2 : Enriched by 2-[^{13}C]-acetate

$\xrightarrow[2\ 1]{}$: $CH_3CO_2^-$; orientation of acetate unit

FIGURE 3. Combined results of 1-^{13}C-acetate and 2-^{13}C-acetate incorporation experiments.

sults of the singly labelled acetate incorporation, and demonstrates that there is indeed an error in the published ^{13}C-nmr assignments (10). There are at least three ways to interchange the assignments for these very similar carbons to make them consistent with the proposed scheme and the labelling results; thus an absolute reassignment of the carbons was not possible from these data alone. No coupling patterns were observed for any other carbons, confirming that C-10 and C-11 are not derived from acetate, and that the incorporation into other carbons was through scrambling reactions.

INCORPORATION OF LABELLED PROPIONATE.—According to the biosynthetic scheme proposed, seven propionate units should be incorporated into rapamycin. It was also possible that 1-[^{13}C]- and 2-[^{13}C]-propionate might label C-11 and C-10; a C_2 unit in rifamycin is derived from two carbons of a propionate unit, the methyl group of which is oxidatively removed after it is incorporated (14). Rapamycin samples enriched by 1-[^{13}C]- and 2-[^{13}C]-propionate were prepared using the same procedures as for the acetate samples, except that 0.5 g/liter was added to the cultures at 2.5 days and an additional 0.25 g/liter was added at 3.5 days of incubation.

In the case of 1-[^{13}C]-propionate, six carbons (C-1, C-7, C-13, C-22, C-26, C-39) showed significant enrichment, as indicated in Table 1. (The chemical shifts of C-7 and C-42 overlap; therefore, it was assumed that the increase in peak intensity is due to enrichment of C-7, not C-42.) Five of these carbons were predicted to be derived from the carboxyl groups of propionate. C-39 corresponds to the carboxyl groups of the proposed C_7 unit and was not predicted to be enriched. Also, C-32 corresponds to the carboxyl of one of the predicted propionates, but no enrichment was observed. These results could

be explained by an error in the nmr assignments of these two carbons (10); they are very similar carbons and the assignments might be interchanged.

One of the carbons predicted to be derived from 1-[^{13}C]-propionate, i.e., C-28, was obscured by the solvent ($CDCl_3$) peaks. In order to determine whether there was any incorporation into this carbon, the ^{13}C-nmr spectra of this sample and an unenriched sample were also obtained in CD_2Cl_2, a very similar solvent. The pattern of the rapamycin peaks was not altered much, and the same six peaks were enriched. In addition, a seventh peak at approximately 77.2 ppm was enriched. Based on the published assignments it was concluded that this peak corresponded to C-28, the seventh predicted propionate carbon.

In the case of 2-[^{13}C]-propionate, seven carbons were enriched. These correspond to the seven carbons predicted by the proposed scheme (C-38, C-25, C-27, C-31, C-33, C-6, C-12). These results confirm the incorporation of the seven propionates and support the possibility that C-32 and C-39 were misassigned (10). It is highly unlikely that C-31 and C-33 could be labelled by 2-[^{13}C]-propionate without C-32 being labelled by 1-[^{13}C]-propionate, nor is there a likely route for C-32 to be exchanged with C-39.

Unlike labelled acetate, labelled propionate did not seem to be scrambled into other non-propionate-derived carbons. Perhaps propionate, once activated, is less likely to enter the citric acid cycle.

CORRECTION OF THE ^{13}C-NMR SPECTRAL ASSIGNMENTS.—The results of the above incorporation studies strongly suggested that there were errors in the published ^{13}C-nmr assignments (10). However, absolute reassignments were not possible from the available data alone. A sample of rapamycin was used to generate HETCOR, COSY, and NOESY maps (Dr. R.P. Borris and Dr. B.H. Arison, personal communication; subject of a future publication). Analysis of the data revealed several errors in the assignments (10), some of which are indicated in Table 1. The assignments of C-3 and C-4 were interchanged, and the peak assigned to C-39 was not assigned to C-32, as suggested by the above incorporation data.

INCORPORATION OF LABELLED METHIONINE.—According to the proposed scheme, the three methoxy carbons in rapamycin are derived from the methyl group of methionine. Unlabelled L-methionine was added to the fermentation medium after 2.5 days of incubation to see if it affects rapamycin production. The addition of 0.5 g/liter methionine caused no marked effect on rapamycin production, while 1.0 and 2.0 g/liter clearly decreased production (approximately 35 and 45% decrease, respectively). These findings were at first surprising because addition of a true precursor is expected to increase production if that precursor is rate-limiting or to have no effect if it is not limiting. However, despite the fact that methionine is the precursor of the side chain of thienamycin and the methoxy group of cephamycin C, it inhibits the production of these antibiotics by *Streptomyces catteleya* (15).

Experiments with L-^{14}C-[methyl]-methionine indicated that addition of 0.5 g/liter of labelled methionine resulted in very high enrichment of rapamycin. The specific activities of the added ^{14}C-[methyl]-methionine and the resulting rapamycin were equal, indicating 100% total incorporation or 33% incorporation at each of the three sites. A rapamycin sample enriched by L-^{13}C-[methyl]-methionine was prepared from cultures which received 0.3 g/liter L-^{13}C-[methyl]-methionine at 2.5 days of incubation. The peaks assigned to C-36, C-50, and C-46, the three methoxy carbons, showed a 15- to 20-fold increase in height, confirming that methionine acts as the methyl donor for these carbons. No other carbons were enriched.

INCORPORATION OF LABELLED GLYCINE.—C-10 and C-11 were not significantly enriched by any of the above precursors. It was thought that glycine might enrich this

two-carbon unit, either after conversion to glycollate (transamination and reduction) or after incorporation into glycerate (via serine). When added to cultures of *S. hygroscopicus* var. *geldanus* var. *nova*, both 1-^{13}C-glycerate and 1-^{13}C-glycollate labelled carbons in geldanamycin which acetate had previously failed to label (16). 1-^{13}C-glycine was found to label a two-carbon unit in saframycin A, a heterocyclic quinone antibiotic synthesized by *Streptomyces lavendulae* (17) but did not label the analogous carbons in naphthyridomycin, a structurally similar antibiotic produced by *Streptomyces lusitanus* (18). In the case of leucomycin biosynthesis by *Streptomyces kitasatoensis*, 1-^{13}C-glycine, 1-^{13}C-glycollate, and 1-^{13}C-glycerate did not enrich the carbons of a previously unlabelled C_2 unit, but this was attributed to the failure of these compounds to enter the cells, because 2-^{13}C-glycerol was shown in later experiments to label these carbons (19).

When added to cultures at 2.5 days of incubation, glycine (up to 2 g/liter) had no effect on rapamycin production. A sample of rapamycin enriched by 1,2-$^{13}C_2$-glycine was prepared from cultures receiving 0.75 g/liter at 2.5 days and 0.25 g/liter at 3.5 days. Only the resonances assigned to the three methoxy carbons (C-36, C-50, C-46) showed an increase in intensity (9- to 10-fold). No other resonances showed any increased intensity over natural abundance or any evidence of ^{13}C-^{13}C coupling. Clearly, glycine is entering the cells but is only incorporated into rapamycin after conversion to the methyl group of methionine (via glycine synthase and tetrahydrofolate reactions).

DISCUSSION

The origins of most of the carbons of rapamycin have been identified, as summarized in Figure 4. The results match those of the predicted scheme quite well, except for the lack of acetate incorporation into C-10 and C-11.

Most of the carbons of the lactone ring of rapamycin are derived from six acetate and seven propionate units, condensed in a "head to tail" fashion. This pattern of incorpora-

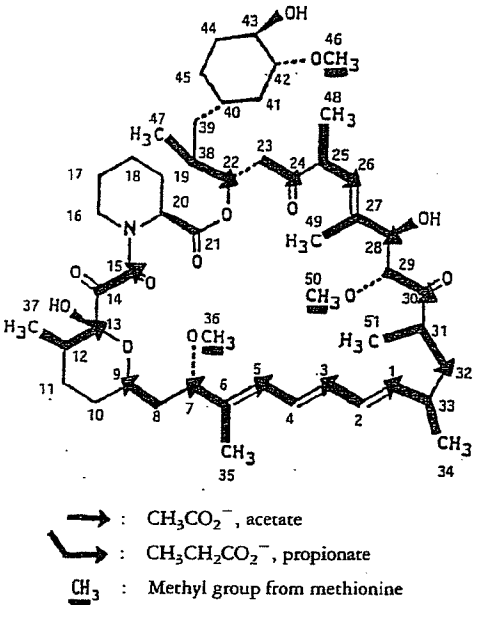


FIGURE 4. Summary of the incorporation results.

tion implies a polyketide pathway mechanism of biosynthesis, as has been found for many acrolides and polyenes. Of note is the fact that the rare α-ketoamide functionality (C-14 and C-15) is indeed derived from acetate, while the high oxidation state of these two carbons and the adjacent carbon might have suggested a carbohydrate origin. The incorporation experiments revealed errors in the published ^{13}C-nmr assignments (10), and these errors were confirmed by further nmr analysis. Those carbons originally misassigned are chemically very similar, and spectroscopic assignments based on similar compounds (or fragments) could easily be wrong.

The seventh predicted acetate unit (C-10 and C-11) was not labelled significantly by any form of acetate, nor by any other precursor tested, and the original of these two carbons remains unknown. It is likely that they are derived from glycollate or glycerate, as has been shown for two-carbon units in geldanamycin and leucomycin (16,19).

The three methoxy carbons of rapamycin were shown to be derived from the methyl group of methionine. Methionine was found to suppress rapamycin production if high concentrations were added to young cultures, but this is consistent with observations for other systems (15). Glycine, while highly enriching the same methoxy carbons after metabolic scrambling, did not decrease rapamycin production.

The substituted cyclohexane moiety (C-39 to C-45) and the heterocyclic ring (C-16 to C-22) were not labelled by any of the precursors tested, as is consistent with their predicted shikimate pathway and pipecolate origins.

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Rapamycin Inhibits Vascular Smooth Muscle Cell Migration

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Abstract

Abnormal vascular smooth muscle cell (SMC) proliferation and migration contribute to the development of restenosis after percutaneous transluminal coronary angioplasty and accelerated arteriopathy after cardiac transplantation. Previously, we reported that the macrolide antibiotic rapamycin, but not the related compound FK506, inhibits both human and rat aortic SMC proliferation in vitro by inhibiting cell cycle–dependent kinases and delaying phosphorylation of retinoblastoma protein (Marx, S.O., T. Jayaraman, L.O. Go, and A.R. Marks. 1995. *Circ. Res.* 362:801). In the present study the effects of rapamycin on SMC migration were assayed in vitro using a modified Boyden chamber and in vivo using a porcine aortic SMC explant model. Pretreatment with rapamycin (2 ng/ml) for 48 h inhibited PDGF-induced migration (PDGF BB homodimer; 20 ng/ml) in cultured rat and human SMC ($n = 10$; $P < 0.0001$), whereas FK506 had no significant effect on migration. Rapamycin administered orally (1 mg/kg per d for 7 d) significantly inhibited porcine aortic SMC migration compared with control ($n = 15$; $P < 0.0001$). Thus, in addition to being a potent immunosuppressant and antiproliferative, rapamycin also inhibits SMC migration. (*J. Clin. Invest.* 1996. 98:2277–2283.) Key words: immunophilin • restenosis • atherosclerosis • FK506 • FKBP12

Introduction

Abnormal vascular smooth muscle cell (SMC)[1] proliferation and migration play major roles in the formation of atherosclerotic plaques, in the development of restenosis after percutaneous transluminal angioplasty (PTCA) and in accelerated arteriopathy after cardiac transplantation (1–5). In normal vessels, the majority of SMC reside in the media, where they are quiescent and possess a "contractile" phenotype (1, 6) characterized by the abundance of actin- and myosin-containing filaments. In disease states, SMC reenter the cell cycle, proliferate, and migrate from the media to the intima (1). PDGF is a potent chemoattractant for SMC (6–8) and is thought to play a major role in stimulating SMC migration in experimental models of vessel injury (9, 10). After vessel injury, intimal SMC demonstrate a synthetic phenotype, secreting extracellular matrix, proteases, growth factors, and cytokines that contribute to further proliferation and migration (1, 5, 6).

Recently, we reported that rapamycin, a macrolide antibiotic, inhibited both human and rat SMC proliferation by blocking cell cycle progression at the G1/S transition (11). This inhibition of cell proliferation was associated with decreases in cell cycle kinase activity and a reduction in the phosphorylation of retinoblastoma protein. We demonstrated that the antiproliferative properties of rapamycin were mediated through binding to the cytosolic receptor, FK506 binding protein (FKBP12) (11). FKBP12 is ubiquitously and abundantly expressed in many cells and tissues and is highly conserved throughout eukaryotic phylogeny (12–16). FKBPs catalyze the *cis–trans* isomerization of peptidyl-prolyl amide bonds of peptides; both rapamycin and FK506 inhibit this rotamase activity. However, the inhibition of rotamase activity is not responsible for the immunosuppressive effects of these agents (17).

In the present study, we demonstrate that rapamycin, but not FK506, blocks rat, porcine, and human SMC migration. The inhibitory effects on migration are competed by molar excesses of FK506, indicating that the effect is mediated through binding to the cytosolic receptor, FKBP12. In rat aortic SMC, the inhibition of rapamycin of both proliferation and migration is mediated through a novel form of FKBP12 which contains three amino acid substitutions compared with the human FKBP12, including a serine substitution for a highly conserved proline at residue 10. The present data indicate that a target of the rapamycin-FKBP12 complex or a downstream effector molecule functions as a critical regulator of SMC migration.

Methods

Reagents. Rapamycin was a gift from Dr. Suren Sehgal (Wyeth-Ayerst Laboratories, Princeton, NJ), FK506 was from Fujisawa (Deerfield, IL), recombinant human PDGF-BB was from Cal Biochem (Stanford, CA).

Cell culture. Rat aortic SMC (passages 8-11) were cultured in DME plus 20% FCS (Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin as previously described (11, 18). The medium was changed every 48 h, and cells were passaged at ~75% confluence. Human aortic SMC (passages 3–5), were obtained from the ascending aorta at the time of cardiac transplantation. The adventitia and connective tissue were removed, the remaining arterial intima and media were cut into 1-cm^2 segments, and placed in culture dishes with collagenase and 15% FCS. Cells were grown in DME medium supplemented with 20% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Aortic SMC were serially passaged before reaching confluence. Cells were identified as smooth muscle by their typical

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1. *Abbreviations used in this paper:* FKBP, FK506 binding protein; GAP, Ras-GTPase–activating protein; PI-3′, phosphatidylinositol-3-OH; PLC, phospholipase C; SMC, smooth muscle cell; PTCA, percutaneous transluminal angioplasty.

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appearance on light microscopy and by immunostaining with anti–smooth muscle α-actin 1A4 (M851; Dako Inc., Carpenteria, CA).

Smooth muscle cell migration assay. Migration was measured using a 48-well chamber (modified Boyden chamber) housing a polycarbonate filter with 8-μm pores (Nucleopore, Cabin John, MD) (19). Each membrane was coated with 0.1 mg/ml of collagen (Vitrogen 100®; Centrix, Santa Clara, CA) in 0.2 M acetic acid for 24 h before each chemotactic assay. For each assay, 27 μl of PDGF-BB (20 ng/ml) in DME plus 0.2% BSA were loaded in quadruplicate wells in the bottom chamber. BSA (0.2% in DME without PDGF) was used as a negative control. SMC were grown to ~ 30% confluence. Rapamycin or FK506 was then added directly to the culture media of some plates, control was without drug. After 48 h of incubation, cells were trypsinized, washed three times with PBS, and counted with a hemocytometer. Cell viability was determined by trypan blue exclusion. To examine the acute effect of rapamycin on SMC migration, rapamycin (100 ng/ml) was added directly to the upper or lower chamber without pretreatment. SMC grown in the absence of rapamycin were loaded in the upper chamber.

Equal numbers of cells (2×10^5/ml) in 50 μl were placed in the top chamber. After 6 h of incubation in 5% CO_2 at 37°C, nonmigrating cells were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with Diff-Quik (Baxter Healthcare Co., Miami, FL). The number of SMC on the lower surface of the filter was determined microscropically by counting five high power (×400) fields of constant area per well. Values are expressed as the percentage of cells migrating in response to PDGF-BB after subtraction of the negative control (DME + BSA). Experiments were performed at least twice using quadruplicate wells. Statistical significance was determined using the unpaired Student's *t* test; $P < 0.01$ was considered a significant result.

Northern analyses. Total RNA was prepared from SMC growing in log phase using the standard guanidinium-isothiocyanate lysis buffer and centrifugation through a cesium chloride cushion as described elsewhere (20). 20 μg of total RNA was size fractionated on a 1% formaldehyde agarose gel, blotted to nitrocellulose, and probed with α-^{32}P-labeled FKBP12 cDNA randomly labeled to a specific activity of 10^9 cpm/μg. The FKBP12 cDNA probe contained the entire coding region of the rabbit FKBP12 (16). Hybridization was at 42°C overnight and final washing at 65°C in 0.2 × SSC. Films were autoradiographed with a single intensifying screen at −70°C for 48 h. Ethidium bromide staining of the 28S ribosomal RNA was used to ensure that equivalent amounts of RNA were loaded in each lane. Northern analyses were repeated at least three times with the same results, a representative blot is shown.

Immunoblots. SMC growing in log phase were washed twice with ice-cold PBS and lysates were prepared using modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Cells were scraped off the bottom of the plates and lysates rocked for 1 h at 4°C. Lysates were clarified by centrifugation on a table top centrifuge for 20 min at 14,000 at 4°C. Protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard. Protein extracts (50 μg) were size-fractionated on 15% SDS-polyacrylamide gels and transferred to nitrocellulose overnight at 45–60 V. Filters were blocked in PBS containing 0.1% Tween 20 (PBS-T), and 5% dry milk for 1 h at 30°C, followed by incubation overnight at 4°C with either an anti-FKBP12 antibody directed against the amino-terminus of FKBP12 (16) or against the carboxy terminus (1/1,000, gift from Dr. Greg Wiederrecht, Merck Research Laboratory). The filters were washed with PBS-T, then incubated with secondary antibody conjugated to peroxidase for 1 h at 4°C and washed; signals were detected using the chemiluminescence detection system (ECL; Bio-Rad) followed by exposure to Kodak XAR film.

Library screening. A rat aortic SMC oligo-dT primed cDNA library in λZAPII was screened using a full-length rabbit FKBP12 cDNA that includes the entire coding region of FKBP12 as described previously (16). A total of 10^6 recombinants were screened at high stringency (final wash was 0.1 × SSC at 65°C). After tertiary screening, five FKBP12 cDNA clones were isolated. The sequence reported in this study was obtained from five independent cDNA clones using the dideoxy chain termination methodology (20).

Explant model. 15 adult Yorkshire albino pigs (body wt 30–35 kg) were randomly assigned to receive either regular pig chow or pig chow plus rapamycin for 7 d. Three animals received 1 mg/kg of rapamycin orally and four animals received 5 mg/kg for 7 d. Animals were euthanized with 10 ml of Sleepaway (26% sodium pentobarbital, 10% isopropyl alcohol, 20% polyethelene glycol; Fort Dodge Laboratories, Fort Dodge, IA). The aortas were perfused in situ with ice-cold physiologic buffer at a pressure of 100 mmHg. The aortas were excised, adventitia and surrounding connective tissue were removed. Aortas were opened by a longitudinal cut and the intima as well as a thin portion of the subjacent media were removed. The media were divided into five 10 × 10-mm^2 segments that were cut into multiple 2 × 2-mm^2 segments and placed in 100-mm culture dishes containing 10 ml of DMEM + 20% FBS. Culture media were changed every 3–4 d. On days 5, 10, and 15, culture dishes were washed with PBS to remove cellular debris, trypsinized briefly with 1.5 ml of trypsin, resuspended in 2 ml of PBS, and cells were counted (eight times per dish) using a hemocytometer. The results are presented in Fig. 5 as mean values±standard error of the mean for eight determinations from each of eight control, and seven rapamycin-treated animals. Cell viability was assessed using the trypan blue exclusion method. Rapamycin levels in whole blood samples were determined by Wyeth-Ayerst Laboratories. Routine histologic examinations of the aortas to assess the effects if any of rapamycin on SMC morphology were performed using hematoxylin and eosin stained sections.

Adhesion assay. Rat aortic SMC were incubated either with or without 20 ng/ml of rapamycin in DMEM supplemented with 10% FBS for 72 h. After 72 h cells were detached from the plates by brief trypsinization in 2 ml of trypsin/EDTA, resuspended in 10 ml DMEM + 10% FBS, and cell number was determined. There were fewer cells in the rapamycin treated samples compared with the control (4.4×10^6 vs 8.8×10^6) due to the antiproliferative effect of rapamycin on smooth muscle cells (11). Four six-well culture plates were precoated with varying concentrations of collagen (from 1 μg/ml to 1,000 μg/ml of Vitrogen). Smooth muscle cells were resuspended in DMEM to give a final concentration of 2.0×10^5 cells/ml. 1 ml of SMC was added to each well. After 2.5 h, nonadherent cells were removed and the number of cells adhering to the bottom of the wells was determined by counting four separate fields of constant area per well. All experiments were performed in triplicate.

Results

Rapamycin as low as 2 ng/ml had a significant inhibitory effect ($P < 0.0001$) on PDGF-BB homodimer-induced rat aortic SMC migration (Fig. 1 *A*). Similarly, rapamycin significantly inhibited PDGF-BB induced human SMC migration (Fig. 1 *B*; $P < 0.0001$). The inhibition of migration was concentration dependent with an $IC_{50} = 2$ ng/ml for both rat and human SMC. In contrast, FK506 (1–500 ng/ml) had no significant effect on migration in response to PDGF-BB (Fig. 1 *A*). The inhibition of migration by rapamycin persisted for at least 72 h in the Boyden chamber assays. By 96 h cell viability was substantially decreased in both control and in rapamycin-treated cultures, preventing assessment of cellular migration. There was no difference in cell viability between the control, FK506, and rapamycin treated cells as assessed by trypan blue staining. No difference in morphology was observed comparing rat SMC from rapamycin-treated and untreated cultures (Fig. 1 *C*). As previously described (11), rapamycin inhibited SMC proliferation, whereas FK506 had no effect (data not shown). However,

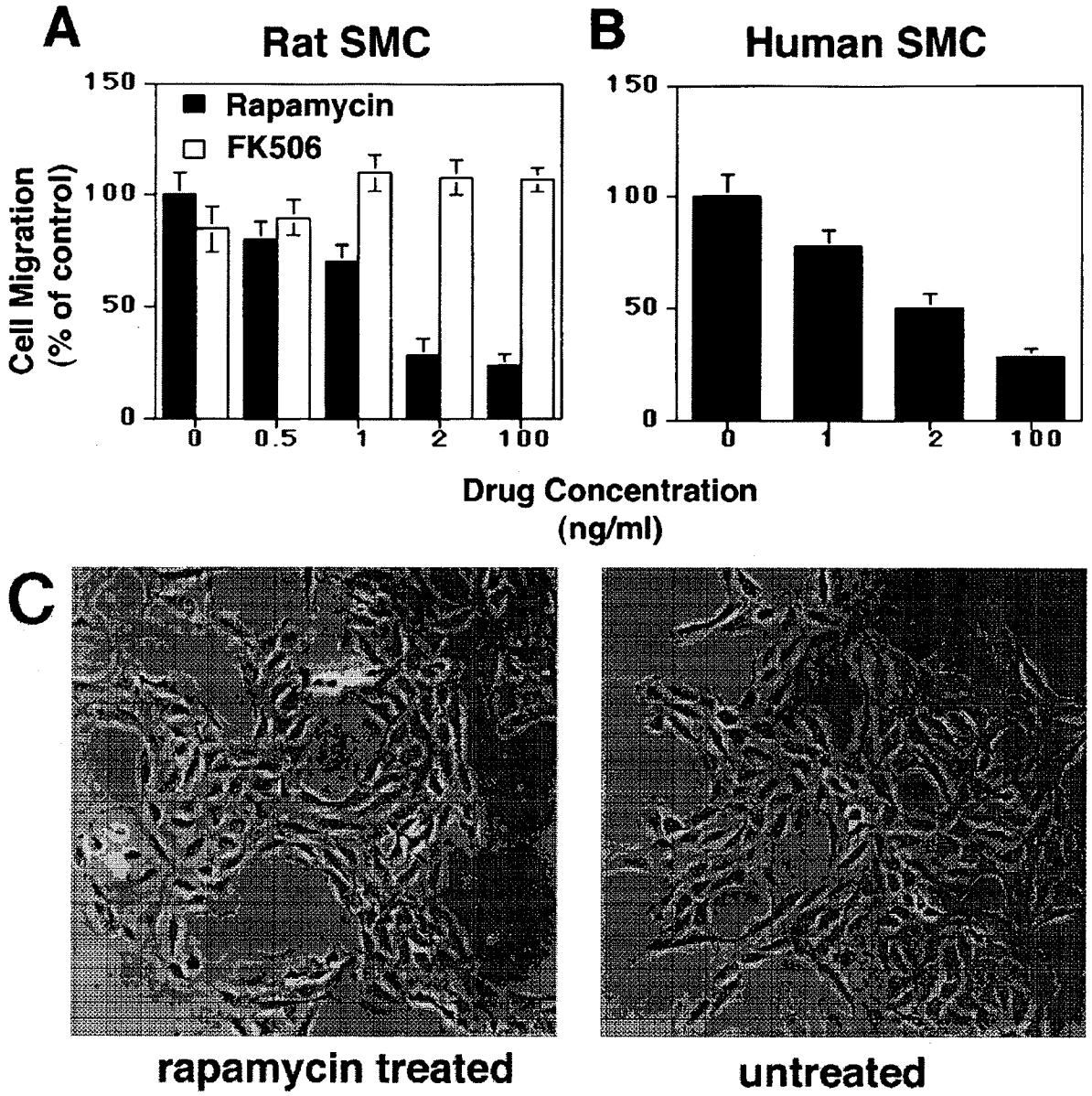

Figure 1. Rapamycin but not FK506 inhibits aortic smooth muscle cell migration. (*A*) Rat aortic SMC were cultured with either rapamycin or FK506 for 48 h and then examined for chemotaxis to 20 ng/ml of PDGF-BB in a modified Boyden chamber. $P = 0.01$ for control vs. 1 ng/ml rapamycin, $P < 0.0001$ for control vs. 2 and 100 ng/ml rapamycin. (*B*) Similar experiments were performed using human aortic SMC cultured with rapamycin. $P < 0.0001$ for each concentration of rapamycin compared with control. There is no significant difference between control and FK506-treated rat or human cells (not shown). The results represent mean values obtained from quadruplicate wells from each of two experiments. Error bars represent standard error of the mean. (*C*) Culturing with rapamycin had no effect on rat SMC morphology; compare control cells to cells cultured for 72 h with rapamycin (20 ng/ml).

equal numbers of cells were used in all migration assays. Thus the reduction in migration did not simply reflect a difference in cell number between control, FK506, and rapamycin treated cells.

Addition of rapamycin (100 ng/ml) to the cells immediately before loading into the upper Boyden chamber (Fig. 2), or incubation with rapamycin (100 ng/ml) for up to 6 h (data not shown) had no effect on migration. Similarly, rapamycin, when added together with PDGF to the lower chamber, had no effect on the ability of PDGF to attract normal SMC (Fig. 2). These results indicate that inhibition of SMC migration by rapamycin requires > 6 h exposure to rapamycin. The inhibition of migration of rat aortic SMC by rapamycin (100 ng/ml) was competitively inhibited by 80±10% by a 100-fold molar ex-

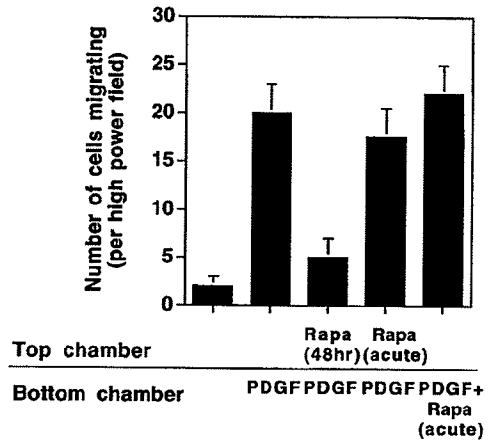

Figure 2. Inhibition of aortic smooth muscle migration requires prolonged exposure to rapamycin. Rat SMC added to the top chamber in the migration assay did not migrate when PDGF-BB was omitted from the bottom chamber (*first bar*). When PDGF-BB (PDGF, 20 ng/ml) was added to the bottom chamber, rat SMC migration was observed (*second bar*). Treatment of rat SMC with rapamycin (Rapa, 100 ng/ml) for 48 h significantly inhibited migration (*third bar*, $P < 0.001$). In contrast, acute administration of rapamycin (100 ng/ml) to the top (*fourth bar*) or bottom (*fifth bar*) chamber had no effect on migration. The y-axis shows the number of cells migrating onto the filter per high power field (see Methods for details).

cesses of FK506, indicating that the inhibition of migration by rapamycin was mediated by the binding to the immunophilin FKBP12, since both rapamycin and FK506 share the same cytosolic receptor.

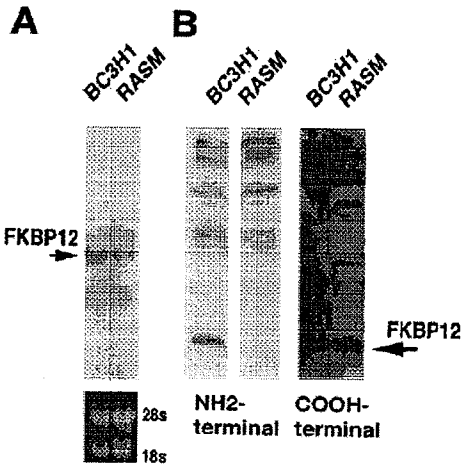

Figure 3. Analyses of FKBP12 mRNA and protein in rat SMC. (*A*) Northern hybridization using a full-length FKBP12 cDNA shows equivalent levels of FKBP12 mRNA (1.5 kb) in a murine muscle cell line (*BC3H1*) and in rat aortic SMC (*RASM*). (*B*) Immunoblot analysis using an anti-FKBP12 antibody (16) that recognizes the amino-terminus of the human, rabbit, and murine FKBP12 shows no detectable FKBP12 protein in rat SMC, whereas an immunoblot using an anti-FKBP12 antibody directed at the carboxyl-terminus of FKBP12 shows a 12-kD protein in rat SMC.

FKBP12 is ubiquitously expressed and highly conserved across species. However, immunoblotting with an antibody that recognizes the amino-terminus of the human, rabbit, and murine FKBP12 did not detect FKBP protein in rat aortic SMC (Fig. 3). In contrast, Northern hybridization using a full-length FKBP12 cDNA revealed high levels of FKBP12 mRNA (1.5 kb) in rat aortic SMC (Fig. 3) and an antibody directed at the carboxyl-terminus of FKBP12 identified a 12-kD protein in rat aortic SMC (Fig. 3). To determine the complete structure of the rat smooth muscle FKBP12, five FKBP12 cDNAs were isolated from a rat aortic SMC library and sequenced (Fig. 4). The rat FKBP12 encodes a 108–amino acid protein whose sequence is 95% identical to the human and rabbit form and 98% identical to the murine sequence. Notably, there is a serine for proline substitution at residue 10 in the middle of the epitope recognized by the amino-terminal FKBP12 antibody.

Rapamycin inhibits intimal thickening in rat aortas after mechanical or alloimmune injury (21) and reduces luminal narrowing following PTCA in porcine coronary arteries by 63% (Gallo, R., A. Padurean, J.H. Chesebro, J.T. Fallon, V. Fuster, A.R. Marks, and J.J. Badimon, manuscript submitted for publication). The latter study showed further that the reduction in luminal narrowing after PTCA was due to inhibition of intimal SMC hyperplasia typically observed in the vessel wall after balloon injury. Reduction in intimal SMC hyperplasia in rapamycin-treated animals presumably reflects the fact that the drug causes a block in G1 to S progression in SMC (11). However, SMC migration is also an important component of the intimal thickening after PTCA. SMC have to migrate from the media into the intima to proliferate and cause luminal narrowing. To assess the in vivo effects of oral rapamycin on SMC migration we examined the ability of cells to migrate out of porcine aortic explants and establish cultures. SMC from control untreated animals began to migrate out of the explanted aortic tissue by day 5 and reached near-confluence on day 15. In contrast, SMC from animals receiving 1 mg/kg of rapamycin did not migrate out of the aortic tissue until day 10, and by day 15 the number of cells was significantly reduced despite the fact that the only exposure to rapamycin for these cells had been in the intact animal (Fig. 5). SMC from animals treated with 5 mg/kg of rapamycin exhibited no migration even by day 15 after explant. Histological examination revealed no morphologic differences between SMC in control versus untreated animals, and trypan blue exclusion tests revealed no increase in cell death in SMC from rapamycin treated animals. Rapamycin levels in whole blood were 30±2ng/ml and 85±5ng/ml for the 1 mg/kg and 5 mg/kg dosing regimens, respectively. The concentration of 30±2ng/ml is comparable to that observed in humans receiving rapamycin after renal transplant, whereas the concentration of 85±5ng/ml would be considered above the recommended therapeutic levels. An assay was done to determine whether rapamycin affected the ability of SMC to bind to collagen. Control rat aortic SMC and cells pretreated with rapamycin using the conditions described above for the Boyden chamber assays were plated on collagen coated culture dishes in DMEM. At 2.5 h there were no significant differences between the rapamycin-treated and the control cells in terms of the number of SMC adhering to collagen-coated and the noncoated culture plates (control 55±3, rapamycin 58±3; P = NS). These results suggest that rapamycin does not affect the ability of smooth muscle cells to adhere to collagen.

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                1
rat FKBP12      ATG GGA GTG CAG GTG GAG ACC ATC TCT TCT GGA GAC GGG CGC ACC TTC CCG
                Met Gly Val Gln Val Glu Thr Ile Ser Ser Gly Asp Gly Arg Thr Phe Pro
human                                   Pro
murine                                  Pro
               18
               AAG CGC GGC CAG ACC TGC GTG GTA CAC TAC ACG GGG ATG CTT GAA GAT GGG
               Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu Asp Gly

               35
               AAG AAA TTT GAC TCC TCT CGG GAC AGA AAC AAG CCT TTT AAG TTT ACA CTA
               Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys Phe Thr Leu
                                                                           Met

               52
               GGC AAG CAG GAG GTG ATC CGA GGC TGG GAA GAA GGG GTA GCC CAG ATG AGT
               Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val Ala Gln Met Ser

               69
               GTG GGC CAG AGA GCC AAA CTG ATA ATC TCC CCA GAC TAT GCC TAT GGA GCC
               Val Gly Gln Arg Ala Lys Leu Ile Ile Ser Pro Asp Tyr Ala Tyr Gly Ala
                                       Thr
                                                   Ser
               86
               ACC GGG CAC CCA GGC ATC ATC CCA CCA CAT GCT ACT CTT GTT TTC GAT GTG
               Thr Gly His Pro Gly Ile Ile Pro Pro His Ala Thr Leu Val Phe Asp Val

               103
               GAG CTT CTA AAA CTG GAA TGA
               Glu Leu Leu Lys Leu Glu
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Figure 4. Comparison of the primary structure of the rat, human, and murine FKBP12. The cDNA and predicted amino acid sequence of the rat FKBP12 compared with human (rabbit is identical to human [16]) and murine FKBP12 (12). The open reading frame encodes a 108–amino acid protein that differs from the human and rabbit FKBP12 at three residues, and from the murine FKBP12 at two residues. The epitope recognized by the amino terminal anti-FKBP12 antibody (16) is denoted by the underlined sequence. These sequence data are available from GenBank, BankIt67151, under accession number U69485.

Discussion

This study demonstrates that rapamycin inhibits rat, porcine, and human SMC migration. We have previously demonstrated that rapamycin has antiproliferative effects on rat and human aortic SMC mediated by binding to FKBP12 and inhibiting cell cycle kinases and delaying phosphorylation of retinoblastoma protein (11). The inhibitory effects of rapamycin on SMC migration are also mediated by its binding to FKBP12. Recently, a mammalian target of the rapamycin-FKBP12 complex (mTOR) has been identified (22, 23). While the immediate downstream target of mTOR has not been identified, a common theme in many cell types is that rapamycin-FKBP12 inhibits p70 S6 kinase (24, 25), cell cycle kinases, hyperphosphorylation of Rb, and up-regulates the cyclin inhibitor p27 (26). Whether the antimigratory actions of rapamycin-FKBP12 in SMC involve any of these signaling molecules remains unproven. However, the present study suggests that rapamycin can be used as a "molecular probe" to dissect signaling pathways regulating SMC migration in much the same way as the drug is being used to gain insight into mechanisms controlling cell growth.

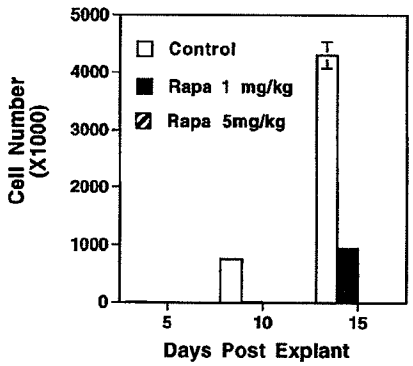

Figure 5. In vivo administration of oral rapamycin inhibits SMC migratory potential. Migratory potential of SMC was examined by determining the ability of cells to move out from porcine aortic tissue samples taken from animals treated with rapamycin compared to control (see Methods for experimental detail). SMC migrating out from the explanted tissue from both control and rapamycin (1 mg/kg per day)–treated animals were able to grow in culture. However, there was a 5-d delay in migration exhibited by SMC in the treated versus control animals resulting in significantly larger numbers of cells (*Cell Number*) in explant cultures from control animals. No SMC migration was observed in aortas from animals treated with the higher concentration of rapamycin (5 mg/kg), even by day 15 after explant. When the standard deviation is small no error bars are seen.

In the present study acute administration of rapamycin had no effect on the rat aortic SMC migration, suggesting that longer exposure to rapamycin (> 6 h) is required. Cellular locomotion required for migration is mediated by the coordinated changes in the polymerization and depolymerization of actin containing microfilaments (27, 28), although the mechanism regulating these changes remain uncertain. Recently, it has been reported that recruitment of signaling proteins including the PDGF receptor and phospholipase C (PLC)-γ and phosphatidylinositol-3-OH (PI-3′) kinase association with the PDGF receptor triggers migration (29). Upon stimulation by PDGF, the PDGF receptor-β autophosphorylates and forms a complex with src homology 2-domain containing proteins such as phosphatidylinositol-specific PLC-γ, Ras-GTPase–activating protein (GAP), and PI-3′ kinase (29). The association of activated PDGF receptor-β with either PLC-γ or PI-3′ kinase promotes migration, whereas association with GAP retards

migration in response to PDGF-β (29). PLC-γ and PI-3′ kinase have been shown to modulate actin assembly (30). Both calcium and polyphosphoinositides have been proposed to regulate actin formation (32). A recent study demonstrated that activation of calcium/calmodulin-dependent protein kinase II (CaM kinase II) is a critical event for the migration of SMC. Inhibiting CaM kinase II blocked migration by > 90%, whereas the inhibition of protein kinase C activation had no significant effect on migration (28). Although mTOR has no apparent PI-3′ kinase activity, its counterpart in yeast, TOR, is a PI-3′ kinase homologue (32).

Moreover, FKBP12 is physically associated with the type I TGF-β receptor, suggesting that FKBP12 may have a direct role in modulating signal transduction mediated by growth factor receptors (33). These observations suggest that one mechanism whereby the rapamycin–FKBP12 complex could block SMC migration might be via inhibition of a growth factor–mediated pathway, possibly one that links migration to cell cycle progression.

The morphology of SMC from treated and untreated cultures (Fig. 1 *C*) and tissue sections was indistinguishable, suggesting that rapamycin does not appear to have an effect on the cytoskeletal components that have been associated with migration. Moreover, rapamycin does not alter the ability of SMC to bind collagen, suggesting that the antimigratory effects of the drug are not due to an inability to interact with the extracellular matrix.

The immunosuppressant drugs rapamycin and FK506 bind to the same site on FKBP12 (34, 35). FKBP12 also binds to naturally occurring cellular targets (36) including three forms of intracellular calcium release channels, calcineurin, and the type I TGF-β receptor (16, 33, 37–40). The immunosuppressant drugs mimic hydrophobic domains found in the cellular targets of FKBP12. The presence of a serine for proline substitution at residue 10 in the rat form of FKBP12 would be expected to introduce a significant structural change at the amino terminus of the rat protein by removing a bend due to the proline residue at this position that is present in the human, rabbit, and murine forms of FKBP12. Thus, it is unlikely that the amino-terminal domain of FKBP12 is involved in mediating the antimigratory effects of the drug.

FKBP12 is physically associated with the skeletal muscle and cardiac ryanodine receptors (37, 38) and the inositol 1,4,5-trisphosphate receptor (39), all of which are calcium release channels located on the sarcoplasmic and endoplasmic reticuli. FKBP12 appears to stabilize the tetrameric structures of these large ion channel complexes and modulates channel gating (37). Moreover, both rapamycin and FK506 interfere with FKBP12's ability to stabilize calcium release channel structures resulting in leaky channels (37). Since the inhibition of migration was specific for rapamycin, and not FK506, it is unlikely that this effect involved modulation of calcium release channels resulting in altered calcium homeostasis.

Our results also demonstrate that administration of rapamycin in vivo inhibits the ability of SMC to migrate. Significantly, this inhibitory effect on migration persists for 2 wk after exposure to rapamycin was stopped. This prolonged inhibitory effect on SMC migration suggests that a 1-wk treatment with oral rapamycin should reduce intimal hyperplasia, even if the stimulus associated with a vascular intervention lasts for up to 3 wk after the procedure.

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